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Post-metabolism impurity profiling of carfentanil, remifentanil, sufentanil, and benzylfentanyl

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ABSTRACT

Carfentanil, remifentanil, and sufentanil are potent fentanyl analogues that are regularly mixed with illicit drugs causing many overdose deaths. Chemical impurity profiling of these drugs is a well-established technique for linking evidence found at a crime scene to other seized samples. The current study aims to expand the application of impurity profiling to metabolized samples to find synthesis specific markers. This is particularly relevant when the drug has been consumed, and no intact material is present at a crime scene. Carfentanil, remifentanil, and sufentanil were synthesized according to the Ugi or 7-step method and benzylfentanyl was produced using the Siegfried method. After in-vitro metabolism with human liver microsomes, the samples were analyzed by gas chromatography-mass spectrometry (GC–MS) and liquid chromatography high resolution tandem mass spectrometry (LC-HRMS/MS). Characteristic markers were found by applying a match criterion approach and principal component analysis (PCA). The precursors 4-ANBP, aniline, and N-phenylacetamide and several metabolites were identified in post-metabolism samples, indicating that specific synthesis information is retained after in-vitro metabolism. The detected levels were in line with concentrations reported in case work. In addition, LDA was applied to maximize discrimination between synthesis methods and to establish likelihood ratios (LRs). Calibrated LR values were in the range of 0.083 to 16 with very low false positive and false negative error rates. In conclusion, the presented work demonstrates the possibility of combining chemical profiling and retrospective biomarker analysis to obtain information about the synthesis method, which could be useful for forensic reconstructions and attribution investigations.

1. Introduction

The misuse of so-called pharmaceutical based agents (PBAs) has increased significantly in the past decade. In particular, the synthetic opioid fentanyl and fentanyl analogues are a great threat to the overall public health, especially in the United States of America (USA) [1]. Fentanyl is a full agonist at the mu-opioid receptor and was first synthesized in the late 1950s as an approximately 50–100 times more powerful analgesic than morphine [2]. Its analogues such as sufentanil, remifentanil, and carfentanil are even more potent [3]. Although first intended to be used as a therapeutic, pain relief drug only, fentanyl analogues quickly emerged in the illicit drug market in the USA where they are regularly mixed with more traditional illicit drugs such as cocaine and heroin [4]. This results in many overdose deaths in what is now known as the opioid crisis. In addition, the potential of fentanyl and fentanyl analogues to be used as a chemical weapon is of increasing concern to national security agencies [5]. A mixture of aerosolized carfentanil and remifentanil was applied by Russian special forces as a

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riot control agent to end the Moscow theatre siege by Chechen militants in 2002 [1,6].

In the field of illicit drugs, chemical impurity profiling is often used to establish a link between seized drugs and a suspect or to link material from different cases. These impurities are often related to the raw materials, the synthesis protocol, and the processing and storage conditions. The analyses are usually performed with liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC–MS) [7]. In general, carfentanil and remifentanil share similar synthetic routes with comparable precursors. One of these routes is the Ugi synthesis and is often referred to as 'Ugi four-compound reaction'. This is a one-pot reaction containing an amine, a carbonyl compound, an isocyanide, and a carboxylic acid [8]. It is relatively straightforward to produce a piperidine precursor with the Ugi reaction, allowing for a 2-step synthesis of car- and remifentanil [9]. Other synthetic routes, such as Strecker [10] and Bargellini [11,12] require more advanced organic chemistry knowledge. Likewise, sufentanil is commonly produced by a multi-step synthesis and often shares similar steps with synthetic routes of other fentanyl analogues [13,14]. Finally, benzylfentanyl, which closely resembles the structure of fentanyl, can be synthesized by the Gupta and Siegfried method, as applied to produce fentanyl [15].

The forensic use of impurity profiling has already been studied extensively for MDMA, amphetamine, methamphetamine, cocaine, and heroin [16–20]. Similarly, three recent chemical impurity profiling studies of fentanyl and its analogues focused on discriminating between synthesis routes. Ovenden et al. established a chemical attribution signature (CAS) for fentanyl batches and identified ten impurities for the Janssen, and five for the Siegfried method [21]. Additionally, four impurities were identified for the fentanyl precursor 4-anilino-N-phenethyl-piperidine (4-ANPP), synthesized according to the Valdez method. Multivariate statistical analysis was performed on liquid chromatography-high resolution mass spectrometry (LC-HRMS) data and successfully applied to differentiate production routes. Mayer et al. identified 160 synthesis-related organic impurities of 3-methylfentanyl using GC–MS and liquid chromatography time-of-flight mass spectrometry (LC-QTOF-MS) [22]. Furthermore, Mörén et al. identified 68 impurities corresponding to either the Strecker, Bargellini, or Ugi synthetic method [23]. The impurities were detected with GC–MS and LC-HRMS and the chemical profiles were classified according to their synthesis route using multivariate statistical analysis [23].

After the release of a chemical warfare agent, attempts will be undertaken to obtain samples from the crime scene to serve as forensic evidence. Environmental samples can be collected, but also biological matrices such as blood and urine from victims can be used as evidence [24]. Especially in cases where no intact chemical is present, biomedical samples can provide essential information. An advantage of biomarkers detected in human biological samples is stability. Biomarkers of the nerve agent sarin could for instance still be identified weeks after exposure in human tissue [25]. Additionally, traces of carfentanil metabolites were found in urine of victims, five days after exposure in the Moscow theatre siege [26]. In such situations, exposed individuals could serve as a valuable source of intelligence for forensic investigators.

In the present study the concepts of chemical profiling and retrospective biomarker analysis were combined to obtain information on the synthetic route after exposure, in a comparable way as previously demonstrated for fentanyl [15]. This study includes one to four batches of three production methods, which were all based on open literature. First, carfentanil, remifentanil, and sufentanil were synthesized according to a 7-step method. Additionally, carfentanil and remifentanil were produced by the Ugi synthesis method. Third, benzylfentanyl was produced from 4-anilino-1-benzylpiperidine (4-ANBP) using the last step of the Siegfried method, as previously illustrated to produce fentanyl [15]. The chemicals were subsequently metabolized in-vitro with human liver microsomes (HLM) to mimic the human metabolic process. GC–MS, liquid chromatography tandem mass spectrometry (LC-MS/MS), and liquid chromatography high resolution tandem mass spectrometry (LC-HRMS/MS) analyses of the pre- and post-metabolism samples were conducted to establish chemical impurity profiles. Possible route-specific markers were identified using both the unsupervised multivariate data analysis method principal component analysis (PCA) and the supervised method linear discriminant analysis (LDA). The present study demonstrates the potential of using biomedical samples for retrieving information about the synthesis route after exposure. This may constitute valuable information when investigating and reconstructing a chemical attack or an overdose case.

2. Materials and methods

2.1. Safety

Due to the extreme potency of carfentanil, remifentanil, and sufentanil, these substances were synthesized by skilled organic chemists in a specially secured laboratory for the production and handling of highly toxic compounds. The antidote naloxone was at hand to be administered directly to mitigate respiratory depression in case of accidental exposure. Chemical analyses were conducted from solution only and low concentrations of psychoactive substances were applied.

2.2. Chemicals

Purities of the chemicals exceeded 97 %. Pooled human liver microsomes (lot #1210097) were obtained from Xenotech (Kansas City, USA). Human plasma was purchased from Sanquin (Amsterdam, The Netherlands). Dichloromethane was purchased from Merck, aniline from Janssen Chimica, d_5-fentanyl from LGC standards, and 4-anilino-1-benzylpiperidine (4-ANBP) from ABCR GmbH. Ethyl-4-oxo-1-piperidinecarboxylate, 1-benzyl-4-piperidone, propionic acid, 4-piperidone monohydrate hydrochloride, glucose-6-phosphate sodium salt (G6P), glucose-6-phosphate dehydrogenase from Leuconostoc mesenteroides (G6P-d lyophilized powder, lot #0000194160), β-nicotinamide adenine dinucleotide phosphate hydrate (NAPD+, lot #SLCG6216), and uridine 5′-diphosphoglucuronic acid trisodium salt (UDPGA) were obtained from Sigma-Aldrich. For LC analysis, formic acid (Fluka), acetonitrile (Biosolve), and MilliQ (Millipak® Express 40) were used.

2.3. Synthesis

All fentanyl analogues were prepared in-house at TNO Defense, Safety & Security (Rijswijk, The Netherlands). The compounds were characterized by NMR, GC–MS, and LC-MS. Because of the labor-intensive methods, a limited number of replicate batches were synthesized, as presented in Table 1. Carfentanil and remifentanil produced by the Ugi route were synthesized by two different scientists. In addition, two batches of carfentanil were produced at least one year apart from the two other carfentanil batches. The purity of the crude products was estimated to be around 50 % and the purified batches had a purity of 82 – 98 %.

Table 1
Overview of applied synthesis methods for carfentanil, remifentanil, sufentanil, and benzylfentanyl.

Compound	Method	Total number of batches	Organic chemists	Product	Purity (%)
Carfentanil	7-step	1	1	purified	90
Carfentanil	Ugi	4	2	crude and purified	49 – 90
Remifentanil	7-step	1	1	purified	82
Remifentanil	Ugi	2	2	crude	50
Sufentanil	7-step	1	1	purified	93
Benzylfentanyl	Siegfried	2	1	purified	94 – 98

Due to the sensitive nature of the synthesis of these compounds, not all synthetic details are provided, but an outline is illustrated in Fig. 1. Only the structures of the starting materials and the intermediate precursors identified in this study are shown. The purity was determined by quantitative NMR by measuring three samples from the same batch. Sufentanil, with a purity of 92.5 % ± 0.4 %, was made according to a 7-step synthetic route using ethyl-4-oxo-1-piperidinecarboxylate as starting material. Benzylfentanyl (purity = 94 % – 98 %) was synthesized from 4-ANBP by the Siegfried method. In addition, carfentanil (purity = 83.5 % ± 0.5 %) and remifentanil (purity = 82.0 % ± 0.02 %) were prepared from 1-benzyl-4-piperidone according to the 7-step synthetic route. Propionic acid, cyanocyclohexene, and aniline were used for the Ugi reaction, which was performed by different organic chemists (purity 50 % – >90 %). The structure of the fourth starting material depended on the desired chemical. N-phenethyl-4-piperidone (R1) was a precursor for carfentanil and methyl (4-oxocyclohexyl)acetate (R2) for remifentanil. Methyl 1-benzyl-4-(phenylamino)piperidine-4-carboxylate (EA6176, purity of 94.9 % ± 0.4 %), which is a simulant for carfentanil, was used as internal standard for benzylfentanyl analysis.

2.4. Human liver microsome incubation

In this study, fentanyl analogues were incubated according to a method described in previous work [15]. For each batch, 3–9 replicate incubated samples were prepared. First, a 0.1 M potassium phosphate buffer with 2.5 mM $MgCl_2$ (pH = 7.4) was prepared. For pre-incubation of remifentanil, sufentanil, and benzylfentanyl, 100 µL of a 1 mg/mL fentanyl analogues solution was used. A lower concentration of 100 µL of 10 µg/mL of carfentanil was applied for safety reasons [2]. The solution of fentanyl analogues was pre-incubated in a Grant-Bio PHMT Thermoshaker at 37 °C and 300 rpm, with 200 µL of 2.5 mg/mL human liver microsomes and 500 µL of the buffer solution. NADPH-regenerating system (NRS) stock was prepared with a final concentration of 1 mM NADP+, 5 mM glucose-6-phosphate, 1 U/mL glucose-6-phosphate dehydrogenase, and 2 mM UDPGA in buffer. The reaction was initiated by adding 200 µL NRS stock solution to the samples. This was then incubated for 72 h at 37 °C and 300 rpm.

Negative controls were prepared by adding 100 µL buffer instead of fentanyl analogue solutions. After incubation, the samples were divided into two fractions of 500 µL for LC and GC analysis. Subsequently, 500 µL acetonitrile was added to the LC fraction to induce protein precipitation. The fractions were centrifuged for 10 min at 14.000 rpm (Eppendorf, 5430R). A final theoretical concentration of 0.9 µg/mL in MilliQ + 0.1 v/v% formic acid with an internal standard concentration of 10 ng/mL was prepared for LC analysis. For the GC fraction, the supernatant was transferred to a glass vial and 400 µL of dichloromethane was added for liquid–liquid extraction. The dichloromethane layer was analyzed with GC–MS.

2.5. Stability remifentanil in blood plasma

To evaluate the stability of remifentanil, 50 µL of 2 µg/mL remifentanil was added to 200 µL blood plasma, which was preheated in a Grant-Bio PHMT Thermoshaker at 37 °C at 300 rpm for 10 min. After 0, 10, 30 min, and every hour until 8 h, 250 µL 0.2 v/v% formic acid in acetonitrile was added to a selection of the samples to stop degradation. The samples were then prepared for analysis with LC-MS/MS. After brief vortexing, the samples were centrifuged for 10 min at 14.000 rpm. Subsequently, 250 µL of the supernatant was transferred to a 1.5 mL vial where 50 µL of a 10 ng/mL d_5-fentanyl solution and 200 µL 0.2 v/v% formic acid in MilliQ were added. A final concentration of 5 ng/mL was analyzed by LC-MS/MS.

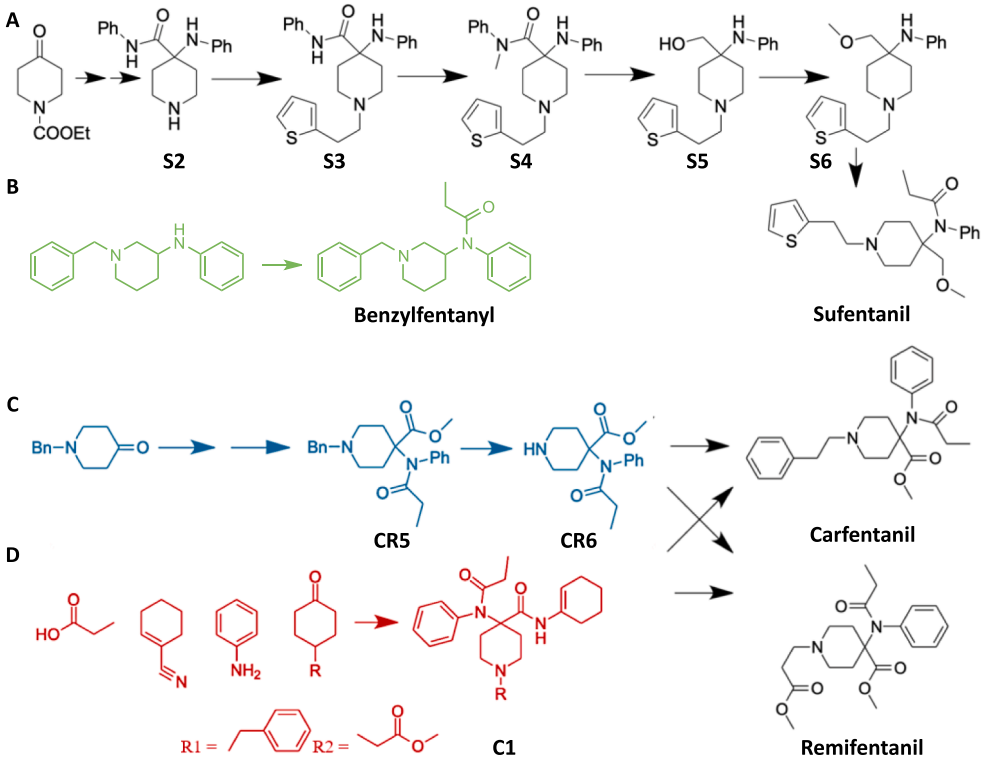


Fig. 1. Reaction scheme of A) 7-step synthesis route of sufentanil (black), B) last step of the Siegfried method for the synthesis of benzylfentanyl (green), C) 7-step synthesis route of carfentanil and remifentanil (blue), and D) Ugi-reaction for carfentanil and remifentanil (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.6. Chemical analysis

2.6.1. GC–MS

Analyses were performed using an Agilent 7890B GC with an Agilent VF-5 ms column (5 % phenylmethyl polysiloxane, 30 m × 0.25 mm × 0.25 μm). The injection volume was 1 μL using an autosampler (Combi Pal, Ctc analytics). Helium was used as carrier gas at a constant flow of 1 mL/min. The GC was operated in splitless mode at 275 °C. The oven temperature was held for 1 min at 40 °C, then increased at 10 °C/min. to 280 °C and maintained at that temperature for 15 min. Detection was performed with an Agilent 5977A MS operating in electron ionization (EI) mode with an ionization potential of 70 eV and a scan range of 50–550 mass units. Compounds were identified with the National Institute of Standards and Technology Mass Spectral Library (NIST MS Search 2.0).

2.6.2. LC-MS/MS

Quantitative analyses were performed with a Waters ACQUITY ultra-high pressure liquid chromatography (UHPLC) system equipped with a Waters ACQUITY UPLC HSS T3 C18 column (1.8 μm, 2.1 x 100 mm). Mobile phase A consisted of MilliQ with 0.2 v/v% formic acid and mobile phase B consisted of acetonitrile with 0.2 v/v% formic acid, using a flow rate of 100 μL/min. Gradient elution started at 100 % eluent A, ramping to 80 % eluent B in 12 min, and holding that solvent composition for 2 min. Prior to the next analysis, the solvent composition was returned to 100 % eluent A within 0.1 min and the system was equilibrated for 2 min. The injection volume was 5 μL. The chromatographic system was coupled to a Waters Xevo TQ-S triple-quadrupole MS detector. The MS operated in positive electrospray ionization (ESI) mode. The capillary voltage was set to 3.5 kV and the cone gas flow was 150 L/h. Data was acquired with selected reaction monitoring (SRM) mode using the transitions, collision energy, and cone energy as depicted in Table 2. Afterwards, the data was analyzed with MassLynx software.

2.6.3. LC-HRMS/MS

Pre-metabolism samples at 0.1–10 μg/mL and post-metabolism samples at 0.009–0.9 μg/mL were analyzed with a Thermo Scientific Ultimate 3000 UHPLC equipped with a Waters ACQUITY UPLC HSS T3 C18 column (1.7 μm, 1.0 x 100 mm). Mobile phase A consisted of MilliQ with 0.2 v/v% formic acid and mobile phase B consisted of acetonitrile with 0.2 v/v% formic acid, using a flow rate of 100 μL/min. Gradient elution started at 100 % eluent A, ramping to 80 % eluent B in 15 min and holding this composition for 7 min. The system was equilibrated for 7.9 min after returning to a 100 % eluent A composition within 0.1 min. The injection volume was 10 μL. The chromatographic system was connected to a Thermo Scientific Q Exactive Plus Orbitrap MS. The MS operated in positive ESI mode and the full mass range was set from 50 to 750 *m/z*. Capillary voltage was set to 3.5 kV and the source temperature was maintained at 320 °C. The relative sheath gas (nitrogen) flow was 35. Data was analyzed with Xcalibur software and Compound Discoverer (version 3.3.1.111) using Chemspider database for tentative identification. The accepted mass error for identification was 5 ppm.

Table 2
Mass spectrometric parameters for analytes and internal standards analyzed by LC-MS/MS.

Analyte	Precursor ion (*m/z*)	Product ions (*m/z*)	Collision energy (eV)	Cone energy (V)
Carfentanil	395.2	335.2, 279.3, 246.1, 133.9	20	17
Remifentanil	377.2	317.0, 228.1, 113.1	15	15
Sufentanil	387.2	335.2, 238.2, 111.0	20	20
Benzylfentanyl	323.1	230.9, 174.0, 90.8	15	10
d_5-fentanyl	342.1	105.0, 188.0	30	20
EA1672	325.1	232.0, 113.0	15	10
Norcarfentanil	291.0	259.1, 230.9, 142.0	10	10
Norfentanyl	233.0	177.1, 149.9	15	40
Remifentanil acid	363.0	303.0, 214.1, 113.1	15	15

2.7. Data analysis

Statistical analysis was performed with Python 3.11.1 using scikit-learn 1.3.0 and lir [27,28]. The code used for this study is based on previous research [29]. The LC-HRMS/MS data was pre-processed by setting a minimum area threshold. For remifentanil samples the threshold was set at a million and for carfentanil samples the threshold was 100,000. In addition, samples were normalized to the area of the internal standard d_5-fentanyl. Before multivariate analysis, a match criterion approach was applied to calculate relative response ratios of selected impurities by dividing the area of an impurity against the area of the fentanyl analogue. Student's *t*-test was performed to calculate the 95 % confidence interval. Subsequently, PCA and LDA were applied to carfentanil and remifentanil samples to reduce the dimensionality and highlight characteristic markers. Sufentanil and benzylfentanyl were not analyzed by multivariate analysis, since only one synthesis method was applied. The model was built using the area of the tentatively identified peaks of 3–12 measurement repetitions of each batch analyzed by LC-HRMS/MS. The varying number of repetitions was selected to have a similar total group size for each synthesis method, regardless of the number of produced batches. The data was normalized using the StandardScaler function, which subtracts the mean and scales the data to unit variance. After pre-processing, PCA was performed to reduce dimensionality of the dataset and to test the discriminating power of the selected impurities. The robustness was evaluated by leave-one-group-out validation. Additionally, LDA was applied as a data-dependent method to maximize discriminative power. Afterwards, Kernel Density Estimation (KDE) was applied to express the likelihood ratio (LR) for assigning unknown samples. The LR is defined as the probability of the evidence given H_1 divided by the probability of the evidence given H_2. For classification of the synthesis route of either carfentanil or remifentanil, the following hypothesis pair was considered:

H_1: The victim has been exposed to carfentanil/remifentanil produced with the 7-step method.
H_2: The victim has been exposed to carfentanil/remifentanil produced with the Ugi method.

Tippett plots were constructed to assess the performance of the LDA model for LR calculations. To prevent extrapolation problems, the values of the LR were limited by imposing empirical lower and upper bounds (ELUB) [30].

3. Results and discussion

3.1. Method optimization and validation

The LC-MS/MS method was optimized in terms of the separation and quantitative analysis of norfentanyl (t_r: 6.37 min), norcarfentanil (t_r: 7.71 min), remifentanil (t_r: 8.30 min), benzylfentanyl (t_r: 8.72 min), d_5-fentanyl (t_r: 9.30 min), EA6176 (t_r: 9.33 min), carfentanil (t_r: 9.75 min), and sufentanil (t_r: 10.04 min). Linear calibration curves were obtained in order to determine the metabolic conversion of the analogues after incubation. These are in the range of 0.5–15 ng/mL for carfentanil, 5–100 ng/mL for remifentanil, 1–20 ng/mL for sufentanil, 0.1–5 ng/mL for norcarfentanil, 0.5–50 ng/mL for benzylfentanyl, and 0.2–10 ng/mL for norfentanyl with R^2 between 0.9969–0.9999. The accuracy of the calibration curves was determined by analyzing quality controls at low, mid, and high concentrations (n = 9–10). The mean values were within

15 % of the actual value. The precisions were below 12 % relative standard deviation (RSD). More details are provided in Section S.1 of the Supplementary data.

The metabolic conversion was assessed after incubation with human liver microsomes. Carfentanil concentration decreased with 85 ± 15 % (stdv., n = 22), sufentanil with 99.9 ± 0.01 % (stdv., n = 2), and benzylfentanyl with 72 ± 11 % (stdv., n = 3). The major metabolite norfentanyl was detected in all samples but at a low concentration of 14.2 ± 2.3 ng/mL. Since the decrease in benzylfentanyl was significantly larger than the formation of norfentanyl, it is hypothesized that a part is degraded or metabolized to another minor metabolite. Further investigation of this effect was beyond the scope of the current study. Also, a norcarfentanil peak was present, but the concentration was below the calibration range preventing quantification. This is likely due to a low recovery because of limited transfer from the polar phase to the organic phase [15]. Remarkably, remifentanil decreased below the detection limit and no main metabolite of remifentanil was observed after in-vitro metabolism. It was hypothesized that remifentanil degraded, therefore a separate stability study was conducted.

The stability of remifentanil was examined in blood plasma (pH = 7.4) during an 8-hour time span. A decline in concentration was observed from 95.9 ± 1.3 ng/mL at 0 h to 19.5 ± 1.0 ng/mL at 8 h as can be seen in Fig. 2A. The drop after 7 h can be explained by the within-sample variability, since each data point reflects a different sample. Interestingly, the addition of 0.2 v/v% formic acid stopped the degradation. The unknown degradation product that was formed was identified as remifentanil acid. Fig. 2B shows the increase of this metabolite from 0.15 ± 0.004 % at 0 h to 2.09 ± 0.08 % at 8 h, relative to the concentration of d_5-fentanyl. This degradation product is in accordance with literature, which describes remifentanil acid as major metabolite due to non-specific metabolism in blood and to a lesser extent the formation of norcarfentanil because of liver metabolism [26,31,32]. Since the degradation was already observed in an alkaline solution (pH > 7) without the presence of a biological matrix, it appears that the stability of remifentanil is more affected by the pH than by specific blood or tissue enzymes [33,34].

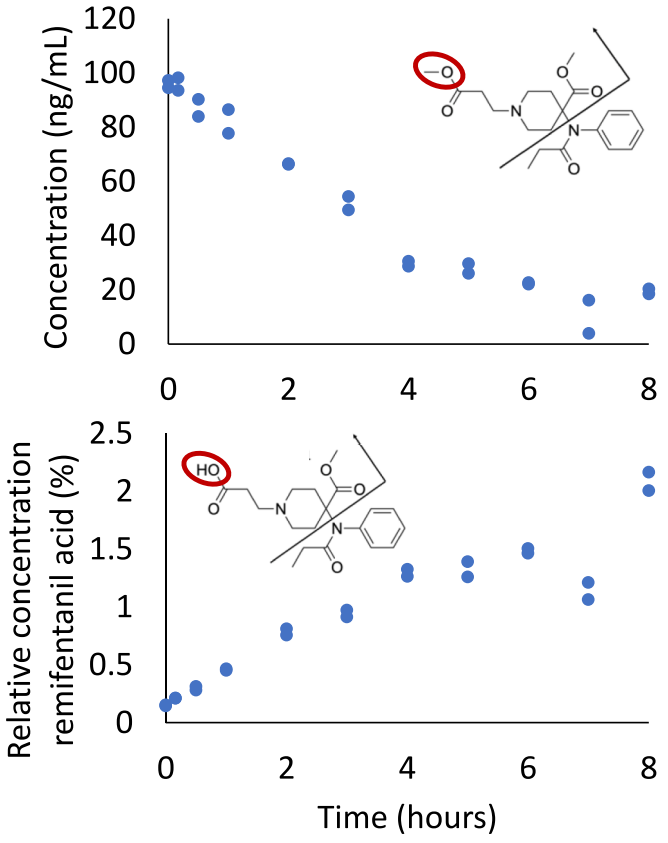


Fig. 2. Stability of remifentanil. A) Concentration remifentanil in blood plasma over time with molecular structure and transition fragment of remifentanil measured by LC-MS/MS. B) Ratio of remifentanil acid with internal standard d_5-fentanyl in blood plasma over time with molecular structure and transition fragment of remifentanil acid.

Fig. 3 shows representative LC-HRMS/MS total ion chromatograms for all fentanyl analogues synthesized with the Ugi and 7-step reaction. The parent compounds sufentanil (t_r: 8.16 min), benzylfentanyl (t_r: 6.80 min), carfentanil (t_r: 7.81 min), and remifentanil (t_r: 6.21 min) are clearly visible. Additionally, some impurties and metabolites are also highlighted: norfentanyl (t_r: 5.39 min), remifentanil acid (t_r: 5.98 min), $C_{17}H_{23}NO_3$ (R.R, t_r: 12.25 min), $C_{12}H_{27}NO_2$ (R.G, t_r: 6.77 min), and $C_{16}H_{35}NO_2$ (R.Q, t_r: 9.94 min). More details on the impurities are given in Section 3.2.

3.2. Impurity profiling

This section elaborates on the identified impurities in sufentanil, benzylfentanyl, remifentanil, and carfentanil samples detected by LC-HRMS/MS. Because of the low levels, no impurities could be identified by GC–MS and only the major compounds sufentanil, benzylfentanyl, and carfentanil were identified. Therefore, in the remainder of this study only the LC-HRMS/MS results are discussed. It should be emphasized that the results are based on a limited sample set and need to be verified by a larger data set in future research.

3.2.1. Sufentanil

A total number of 2800 and 1350 markers were tentatively identified with the ChemSpider database in pre- and post-metabolism sufentanil samples, respectively. For all markers, the mass and chemical formula were known and for approximately a third of the compounds, the database match included a chemical name. From this list a selection was made based on compounds related to the synthesis method. Table 3 shows an overview of the most relevant impurities detected by LC-HRMS/MS in sufentanil samples synthesized according to the 7-step method. The impurities were present in all repeated measurements of only one synthesized batch. The tentative structures are based on comparison with reference databases or literature. In the pre-metabolism samples, a total of six impurities were identified. What stands out in the table is the presence of five precursors of the 7-step method in the pre-metabolism samples (S2-S6, also shown in Fig. 1). In addition, the sixth impurity, thiofentanyl (S.G) is structurally similar to sufentanil and is most likely a synthesis by-product. Although only one synthesis route was examined, the presence of multiple precursors of the 7-step method is very characteristic and not expected in sufentanil synthesized using other precursors. However, to develop a full picture of possible impurities, additional studies will be needed that evaluate more synthesis methods.

In the post-metabolism samples, a total of nine impurities were identified, including two precursors. It is worth noting that precursor S5 could also be formed by metabolism of sufentanil via amide hydrolysis and demethylation. Interestingly, the other seven impurities are metabolites of the precursors S5 and S6. They can be formed because of N-dealkylation, (di)hydroxylation, or glucuronidation. These results indicate that characteristic synthesis information is retained after metabolism. This is a rather surprising outcome, since a batch with a high purity of 92.5 % was examined.

3.2.2. Benzylfentanyl

A total number of 2500 markers were tentatively identified with the ChemSpider database in pre- and post-metabolism benzylfentanyl samples. For 700 compounds the database match included a chemical name. Table 4 lists the most important impurities, related to the synthesis method, detected by LC-HRMS/MS in benzylfentanyl samples

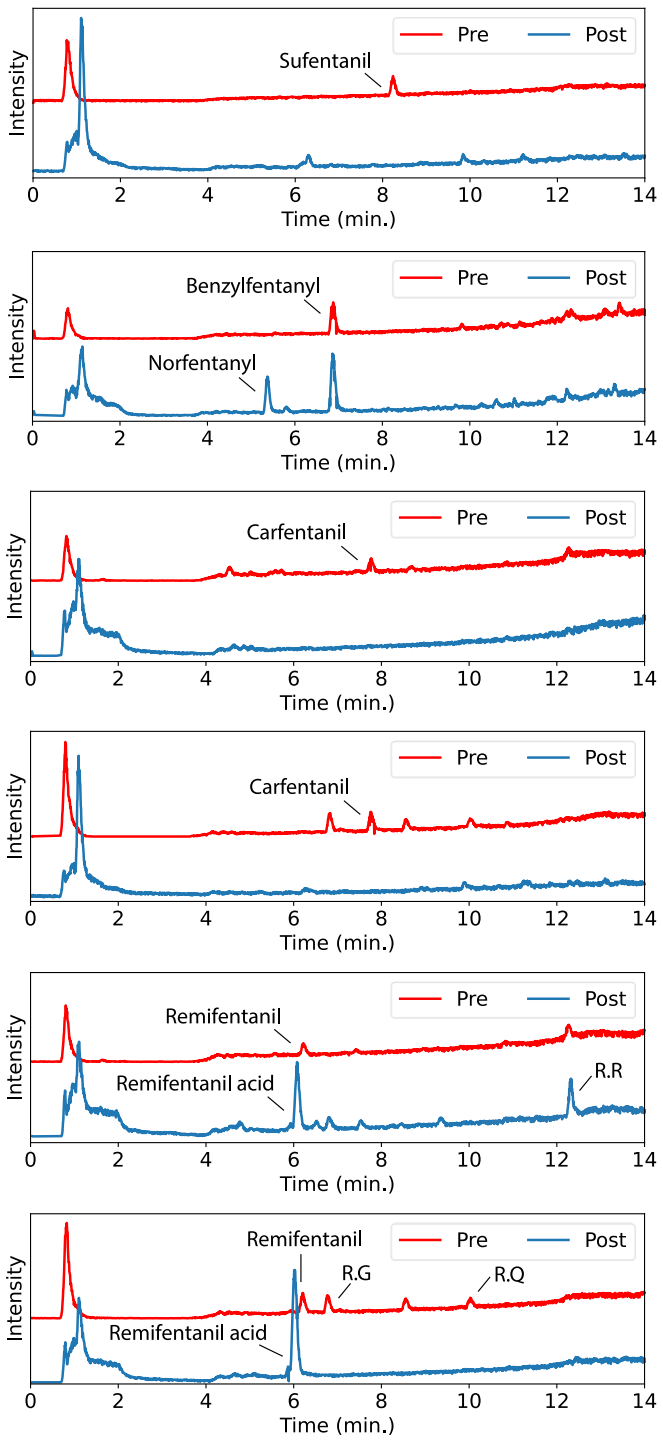


Fig. 3. LC-HRMS/MS total ion chromatograms pre-metabolism (red) and post-metabolism (blue) for A) Sufentanil, B) Benzylfentanyl, C) Carfentanil synthesized with Ugi-reaction, D) Carfentanil synthesized with 7-step reaction, E) Remifentanil synthesized with Ugi-reaction, and F) Remifentanil synthesized with 7-step reaction. Visible impurities and metabolites have been highlighted: norfentanyl, remifentanil acid, C17H23NO3 (R.R), C12H27NO2 (R.G), and C16H35NO2 (R.Q). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

synthesized using the Siegfried method. The impurities were present in all repeated measurements of two synthesized batches. In the pre-metabolism samples, a total of three impurities were tentatively identified including the precursor 4-ANBP (B.F). The structurally similar marker 4-ANPP was also found to be indicative of fentanyl synthesized by the Siegfried method [15]. The authors demonstrated that the marker was present in much higher amounts in the fentanyl samples synthesized by the Siegfried method compared to the Gupta method that produces fentanyl using a single reaction vessel. In addition, benzyl isobutyl ketone (B.A) was identified as a marker. This compound has not been used for other industrial or pharmaceutical applications and may be specific for the synthesis of benzylfentanyl. Also, benzyl acrylfentanyl (B.I) was identified, which is structurally similar to benzylfentanyl.

As expected, norfentanyl was detected in post-metabolism samples. Furthermore, a total of eleven impurities were identified after in-vitro metabolism, including all markers that were detected in pre-metabolism samples. Of interest here is the presence of both the intact precursor 4-ANBP and its metabolites B.G and B.H. Likewise, benzyl acrylfentanyl (B.I) is also present as an intact marker in the post-metabolism samples and in its various metabolic forms. The remaining six impurities (B.B, B.D, B.E, B.K, B.L, and B.M) are (di)hydroxylation, glucuronidation, or N-dealkylation metabolites of benzyl acrylfentanyl (B.I). Similar to the results obtained for sufentanil, this data also show that characteristic synthesis information is potentially retained after metabolism. In this case the purity of the batches was even higher (94–98 %).

3.2.3. *Remifentanil*

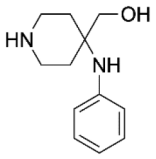
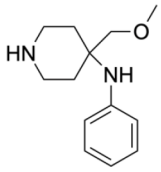
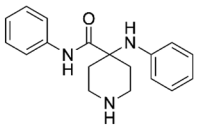
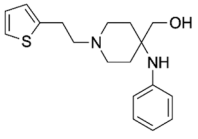
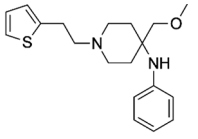
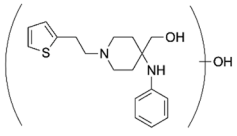
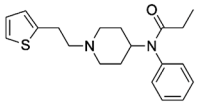
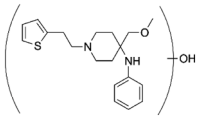
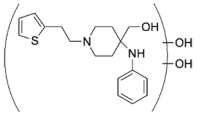
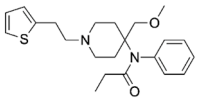
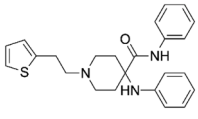
A total number of 1000 impurities were tentatively identified with the ChemSpider database in pre-metabolism remifentanil samples. The chemical name was included for 350 compounds. In addition, 1500 markers were identified in the post-metabolism samples. The database match included a chemical name for 600 compounds. Table 5 provides an overview of the most important impurities detected by LC-HRMS/MS in remifentanil samples synthesized by the 7-step and Ugi method. The impurities were present in all repeated measurements of two batches for the Ugi method synthesized by different scientists and one batch for the 7-step method. A more detailed table with 18 additional markers is shown in Section S2 of the Supplementary data. For these additional impurities no tentative structure could be established, so only the chemical formula and mass are provided. As expected, norcarfentanil was detected in the post-metabolism samples and remifentanil acid was detected in all samples. In the pre-metabolism samples, a total of nine impurities were identified. Seven impurities were detected in samples produced by the 7-step synthetic route, while one marker was detected in the samples synthesized by the Ugi-method, and one was present in both types of samples.

In post-metabolism samples, a total of thirteen impurities were identified. Four impurities were specific for the 7-step synthetic route, nine markers were only detected in the samples synthesized by the Ugi-method, and one was present in both types of samples. Only the precursor aniline could be tentatively identified in the post-metabolism samples as marker for the Ugi-reaction. It should be noted that this impurity is a commonly used precursor for a wide range of chemicals. However, it is toxic to humans therefore it is avoided in prescription drugs [35,36]. Interestingly, this precursor was also detected in post-metabolism fentanyl samples synthesized by the Gupta and Siegfried method [15]. Since most impurities could not be tentatively identified by comparison with databases, further work can focus on elucidating the structures by NMR analysis or comparison with reference standards.

3.2.4. *Carfentanil*

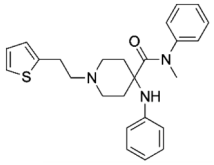
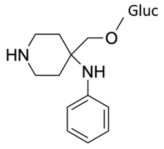
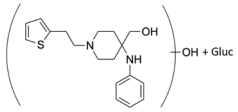
A total number of 8000 and 1800 markers were tentatively identified with the ChemSpider database in pre- and post-metabolism carfentanil samples, respectively. For slightly more than a third of the compounds, the database match included a chemical name. Based on this, a selection was made with compounds related to the synthesis method. Table 6 provides an overview of the most important impurities detected by LC-HRMS/MS in carfentanil samples synthesized according to the 7-step and Ugi-methods. The impurities were present in all repeated measurements of four batches for the Ugi method synthesized by different

Table 3
Pre- and post-metabolism impurities detected by LC-HRMS/MS for sufentanil synthesized according to the 7-step method. Chemicals are sorted by their mass.

Ref.	Name/biotransformation	Chemical formula	*m/z* [M + H]$^+$	t_r (min)	Pre, post	Tentative structure
S.A	N-dealkylation S5†	$C_{12}H_{18}N_2O$	207.150	2.36	Post	
S.B	N-dealkylation of S6†	$C_{13}H_{20}N_2O$	221.165	4.75	Post	
S.C	S2†	$C_{18}H_{21}N_3O$	296.176	7.17	Pre, post	
S.D	S5†	$C_{18}H_{24}N_2OS$	317.168	6.26	Pre, post	
S.E	S6†	$C_{19}H_{26}N_2OS$	331.184	7.25	Pre	
S.F	Hydroxylation of S5†	$C_{18}H_{24}N_2O_2S$	333.164	5.63, 4.42	Post	
S.G	Thiofentanyl*	$C_{20}H_{26}N_2OS$	343.184	6.91	Pre	
S.H	Hydroxylation of S6†	$C_{19}H_{26}N_2O_2S$	347.179	5.88	Post	
S.I	Dihydroxylation of S5†	$C_{18}H_{24}N_2O_3S$	349.159	4.36, 4.26, 4.50	Post	
S.J	Sufentanil* [GC]	$C_{22}H_{30}N_2O_2S$	387.210	8.16	Pre, post	
S.K	S3†	$C_{24}H_{27}N_3OS$	406.195	8.80	Pre	

(*continued on next page*)

Table 3 (*continued*)

Ref.	Name/biotransformation	Chemical formula	*m/z* [M + H]$^+$	t_r (min)	Pre, post	Tentative structure
S.L	S4†	$C_{25}H_{29}N_3OS$	420.211	8.86	Pre	
S.M	N-dealkylation + glucuronidation of S5† OR N-dealkylation + demethylation + glucuronidation of sufentanil†	$C_{21}H_{31}N_2O_8$	439.208	4.76	Post	
S.N	Hydroxylation + glucuronidation of S5†	$C_{24}H_{32}N_2O_7S$	493.201	5.93	Post	

*Identification with Compound Discoverer; †Identification and tentative structure determined by comparison with literature; [GC]Compound also detected using GC–MS.

scientists and one batch for the 7-step method. A more detailed table with 18 additional impurities without structural identification is shown in Section S3 of the Supplementary data. In the pre-metabolism samples, a total of sixteen impurities were detected. In addition, norcarfentanil was detected which is not only a metabolite, but also a precursor in the 7-step synthesis method of carfentanil. Three impurities were specific for the 7-step synthetic route and thirteen markers were only detected in the samples synthesized by the Ugi-method. What is striking about these results is that different precursors were found for both synthetic methods, indicating the potential of attribution studies. The 7-step synthesis intermediate benzyl carfentanil (CR5, Ref. C.E) was only detected in the 7-step samples and the precursors of the Ugi-method aniline and C1 (C.G) were present in a considerable higher concentration in the Ugi-batches. Interestingly, eight other impurities with *m/z* 218.154, 222.149, 258.149, 290.176, 319.202, 324.208, 329.202, and 367.202 (C.H, C.I, C.L, C.M, C.P, C.Q, C.R, C.S, and C.W as shown in Table 6) indicative of the Ugi-method were also found by Mörén et al. as selective markers for this synthesis route [23].

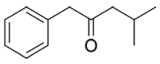
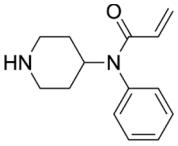
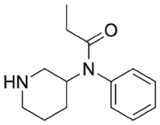
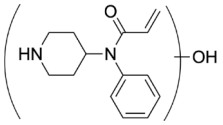
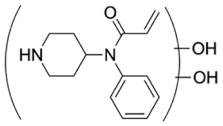
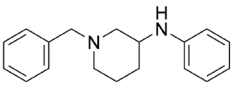
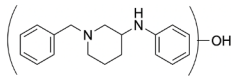
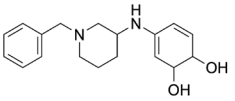
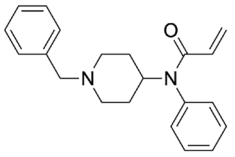
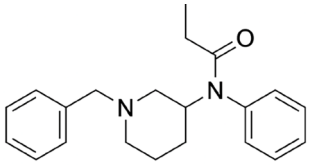
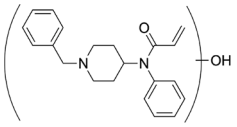
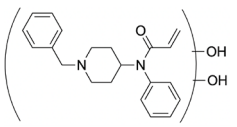
A total of fifteen markers were found in the post-metabolism samples, including seven impurities that were also found in pre-metabolism samples. Five of these were also identified by Mörén et al. [23]. Remarkably, one of these impurities was only present in the post-metabolism samples in the current study. It may be that the impurity concentration was below the detection limit in the pre-metabolism samples. Two impurities were specific for the 7-step synthetic route and thirteen markers were only detected in the samples synthesized by the Ugi-method. Closer inspection of Table 6 shows N-phenylacetamide (C.B) as post-metabolism marker of the Ugi-method, which was also identified in earlier studies as N-acetylation metabolite of the fentanyl precursor aniline [15,37]. Similar to aniline itself, this compound is toxic due to interference with the oxygen transport by hemoglobin [36]. Although it has been used as an analgesic in the past, it has been replaced by the commonly used aniline derivative and pain killer acetaminophen (paracetamol). Therefore, it is not anticipated to find N-phenylacetamide in regular prescription drugs. Additionally, acetaminophen (C.C) was identified, which is a hydroxylation product of N-phenylacetamide [36]. This compound is often mixed with illicit drugs and is for this reason not a specific marker for carfentanil synthesis. Finally, (di)hydroxylation metabolites of pre-metabolism impurities were detected. For some impurities, the intact impurity and its metabolite were both detected in the post-metabolism impurity profile. Although a relatively low concentration was analyzed, still a lot of characteristic impurities were detected in both crude and purified batches.

3.3. Match criterion approach

Within forensic investigations it is often difficult to ascertain the exact exposure concentration. Since, the absolute amounts of the impurities are dependent on this level, normalization is required to correct for this. Match criterion approaches are commonly used for the comparison of impurity profiles of various sources [38]. Table 7 shows the responses of three distinctive impurities after in-vitro metabolism of remifentanil relative to the response of remifentanil acid, due to the absence of remifentanil itself. The results are presented of six repeated measurements of one batch of remifentanil produced by the 7-step method and in total six measurements of two batches of remifentanil produced by the Ugi method. The impurities aniline and R.H ($C_{12}H_{14}N_2O_2$, *m/z* 219.113) are characteristic for the Ugi synthesis method and R.G ($C_{12}H_{27}NO_2$, *m/z* 218.212) is distinctive to the 7-step method. It is apparent from this table that there are no overlapping ranges. A sensitive value is the ratio of R.H/R.G which is 33,000 times larger for the Ugi method compared to the 7-step method. Also, the ratio aniline/R.G gives a value that is 240 times higher for the Ugi method compared to the 7-step method. This approach can serve as a relatively straightforward tool to discriminate between synthetic routes, although more batches should be analyzed to verify these results.

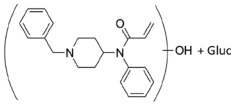
Additionally, characteristic relative responses were found for carfentanil impurities, after in-vitro metabolism. Table 8 shows the 95 % confidence intervals of six characteristic markers for the 7-step or Ugi method. The results are presented of nine repeated measurements of one batch of carfentanil produced by the 7-step method and in total 19 measurements of four batches of carfentanil produced by the Ugi method. The marker C.Y was characteristic for the 7-step method and all other markers were distinctive to the Ugi method. Some overlap was visible in responses of C.Y for the 7-step and the Ugi method. Nonetheless, a valuable ratio would be C.Y/C.T which is 88 times larger for the 7-step method compared to the Ugi method. The 95 % confidence intervals of more impurities of remifentanil and carfentanil can be found in Section S4 of the Supplementary data. It should be noted that the variation in the level of impurities present in the 7-step batch is likely larger in situations where more than one batch is considered.

Table 4
Pre- and post-metabolism impurities detected by LC-HRMS/MS for benzylfentanyl synthesized using the last step of the Siegfried method.

Ref.	Name/biotransformation	Chemical formula	*m/z* $[M+H]^+$	t_r (min)	Pre, post	Tentative structure
B.A	Benzyl isobutyl ketone*	$C_{12}H_{16}O$	177.128	6.85	Pre, post	
B.B	N-dealkylation of benzyl acrylfentanyl†	$C_{14}H_{18}N_2O$	231.150	5.25	Post	
B.C	Norfentanyl†	$C_{14}H_{20}N_2O$	233.165	5.39	Post	
B.D	N-dealkylation + hydroxylation of benzyl acrylfentanyl†	$C_{14}H_{18}N_2O_2$	247.145	4.78, 6.91	Post	
B.E	N-dealkylation + dihydroxylation of benzyl acrylfentanyl†	$C_{14}H_{18}N_2O_3$	263.140	5.25, 5.51	Post	
B.F	4-ANBP†	$C_{18}H_{22}N_2$	267.186	6.39	Pre, post	
B.G	Hydroxylation of 4-ANBP†	$C_{18}H_{55}N_2O$	283.181	6.24	Post	
B.H	Dihydrodiol formation of 4-ANBP†	$C_{18}H_{24}N_2O_2$	301.192	5.55	Post	
B.I	Benzyl acrylfentanyl*	$C_{21}H_{24}N_2O$	321.197	6.67	Pre, post	
B.J	Benzylfentanyl* [GC]	$C_{21}H_{26}N_2O$	323.212	6.80	Pre, post	
B.K	Hydroxylation of benzyl acrylfentanyl†	$C_{21}H_{24}N_2O_2$	337.192	5.99, 6.30	Post	
B.L	Dihydroxylation of benzyl acrylfentanyl†	$C_{21}H_{24}N_2O_3$	353.189	6.08, 6.54	Post	

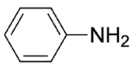
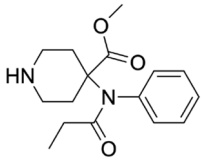
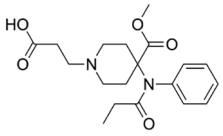
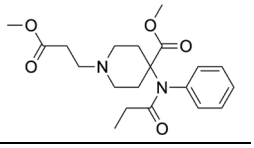
(*continued on next page*)

Table 4 (*continued*)

Ref.	Name/biotransformation	Chemical formula	*m/z* [M + H]$^+$	t_r (min)	Pre, post	Tentative structure
B.M	Hydroxylation + glucuronidation of benzyl acrylfentanyl†	$C_{27}H_{32}N_2O_7$	513.224	5.76	Post	

*Identification with Compound Discoverer; †Identification and tentative structure determined by comparison with literature; [GC]Compound also detected using GC–MS.

Table 5
Pre- and post-metabolism impurities detected by LC-HRMS/MS for remifentanil synthesized according to the 7-step and Ugi-method.

Ref.	Name/bio-transformation	Chemical formula	*m/z* [M + H]$^+$	t_r (min)	Pre, post	Method	Tentative structure
R.A	Aniline	C_6H_7N	94.065	1.46	Post	Ugi	
R.B	Norcarfentanil (CR6)*	$C_{16}H_{22}N_2O_3$	291.170	5.87	Post	7-step, Ugi	
R.C	Remifentanil acid†	$C_{19}H_{26}N_2O_5$	363.191	5.98	Pre, post	7-step, Ugi	
R.D	Remifentanil*	$C_{20}H_{28}N_2O_5$	377.207	6.21	Pre, post	7-step, Ugi	

*Identification with Compound Discoverer; †Identification and tentative structure determined by comparison with literature.

3.4. Chemometric comparison

3.4.1. Remifentanil

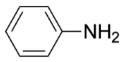
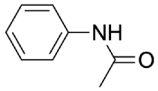
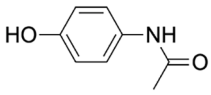
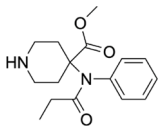
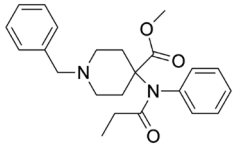
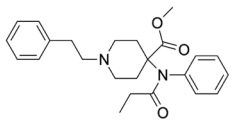
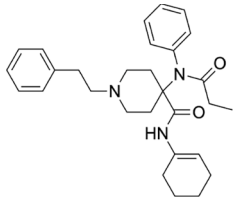
Multivariate data analysis was applied to LC-HRMS/MS data to differentiate between the 7-step and Ugi synthetic route. First, PCA was applied to reduce the dimensionality and to visually identify discriminating impurities after in-vitro metabolism. Fig. 4A shows the PCA score plot of the first two principal components (PCs) for post-metabolism remifentanil samples. The first PC accounts for 61 % of the variance. Excellent separation of the two synthesis methods is visible, which is fully achieved by the first principal component and predominantly caused by impurities from the Ugi method (Fig. 4B). Of interest here are the characteristic impurities R.G, R.H, and aniline (R.A) that were also considered for the match criterion approach. Likewise, good separation was observed for the pre-metabolism samples (section S5 in the Supplementary data). As expected, all impurities contribute to the separation, since most markers were either found in the Ugi batch or in the 7-step samples, without overlapping responses. The robustness of the PCA model was demonstrated by its consistent explained variance even when a sample was excluded. The leave-one-out validation plots are provided in Section S5 of the Supplementary data.

Additionally, LDA was applied to achieve maximum discrimination between the two distinct groups. Since the number of impurities were in the same range as the number of samples, the first six principal components of PCA (94 % of the variance) were used as input for the LDA [39]. Fig. 5 shows the LDA scores and corresponding LRs with ELUB bounds for post-metabolism samples of remifentanil for both synthesis routes. Perfect separation was visible with false positive and false negative error rates near zero. The uncalibrated LR values without ELUB bounds were in the range of 10^{-200} to 10^{100}. Since, a limited number of samples were analyzed, the corrected LRs were much smaller, in the range of 0.17 to 6.0. Section S5 of the Supplementary data elaborates on the Tippett plots and pre-metabolism results. The LDA plot of the pre-metabolism samples also shows separation of the two synthesis methods. No misleading evidence was calculated for the uncorrected and ELUB LR distribution. The LR values without ELUB bounds were in the range of 10^{-50} to 10^{250} and the corrected LRs were between 0.090 and 6.4. Overall, these results indicate that if a positive LR is obtained for an unknown (biomedical) sample, the profile is more probable when the victim has been exposed to remifentanil produced according to the 7-step synthetic route (H_1), than when the victim has been exposed to remifentanil synthesized with the Ugi method (H_2).

3.4.2. Carfentanil

In accordance with the remifentanil results, the application of PCA to carfentanil samples after in-vitro metabolism also provided separation of the 7-step and Ugi synthesis methods. Fig. 6A presents the PCA score plot of the first two PCs for post-metabolism carfentanil samples. The first PC accounts for only 34 % of the variance and the second PC for 25 %. It is worth noting that both components are required for separation, where the first PC is mostly dominated by C.P which is a characteristic marker for the Ugi method. The second component is predominantly composed of C.N, C.T, C.U, and C.V, which are also distinctive Ugi markers. The various batches are clearly distinguished. The Ugi samples in the top left are the purified samples, the samples in the left bottom are unpurified samples, and the samples in the right bottom are two other

Table 6
Pre- and post-metabolism impurities detected by LC-HRMS/MS for carfentanil synthesized according to the 7-step and Ugi method.

Ref.	Name/biotransformation	Chemical formula	*m/z* $[M+H]^+$	t_r (min)	Synthetic route	Pre, post	Tentative structure
C.A	Aniline*	C_6H_7N	94.065	6.85	Ugi	Pre	
C.B	N-phenylacetamide [15,37]	C_8H_9NO	136.073	4.93	Ugi	Post	
C.C	Acetaminophen [37]	$C_8H_9NO_2$	152.071	7.57	Ugi	Post	
C.D	Norcarfentanil (CR6)	$C_{16}H_{22}N_2O_3$	291.170	5.87	7-step, Ugi	Pre, post	
C.E	Benzyl carfentanil (CR5)	$C_{23}H_{28}N_2O_3$	381.218	7.30	7-step	Pre	
C.F	Carfentanil* [GC]	$C_{24}H_{30}N_2O_3$	395.233	7.81	7-step, Ugi	Pre, post	
C.G	C1*	$C_{29}H_{37}N_3O_2$	460.296	13.03	Ugi	Pre	

*Identification with Compound Discoverer; †Identification and tentative structure determined by comparison with literature; [GC]Compound also detected using GC–MS.

Table 7
Characteristic relative responses of remifentanil impurities for the 7-step and Ugi-method detected after in-vitro metabolism. The 95 % confidence interval is shown (n = 6). Responses are relative to the peak area of remifentanil acid.

Impurity	7-Step (%)	Ugi (%)
Aniline	0–1.01	16.1–17.8
R.G	0.012–0.037	0.0002–0.0077
R.H	0–0.01	17.3–30.6

Table 8
Characteristic relative responses of carfentanil impurities for the 7-step (n = 9) and Ugi-method (n = 19) detected after in-vitro metabolism. The 95 % confidence interval is shown. Responses are relative to the peak area of carfentanil.

Impurity	7-Step (%)	Ugi (%)
C.K	0.8–6.8	18.7–67.4
C.N	1.4–3.5	5.7–49.8
C.P	1.2–3.1	11.8–22.3
C.T	1.1–3.1	3.6–54.4
C.V	1.4–3.7	5.5–39.5
C.Y	34–315	0 – 57

batches of unpurified samples synthesized by two different chemists. Therefore, the separation in the PCA is largely due to differences in purification. These results indicate that slight differences in purification can provide characteristic information. An advantage of this is that the impurity profiles can be used to link a case sample to a specific production facility. Additionally, discrimination was observed for the pre-metabolism samples and the leave-one-out validation plots show robustness of the PCA model (Section S6 of the Supplementary data). Interestingly, consistent with the remifentanil results, aniline (C.A) is an important marker for the pre-metabolism carfentanil samples synthesized by the Ugi method. It is somewhat surprising that C.I ($C_{13}H_{19}NO$, *m/z* 222.149) and C.W ($C_{22}H_{26}N_2O_3$, *m/z* 367.202) were found to be discriminating markers for the second principal component. These markers were previously identified as indicative of the Ugi method but were found in similar levels in samples synthesized by the 7-step in this study. It may be possible that these markers are not specific for a synthesis route. An alternative explanation for this result is that the compounds are isomers since no structural identification is provided.

To accomplish maximum separation, LDA was constructed using the first six PCs of the PCA (81 % of the variance). Fig. 7 illustrates the LDA distribution and corresponding LR scores with ELUB bounds for post-metabolism samples of carfentanil synthesized with the 7-step and Ugi method. The KDEs in the LDA plot show some overlap, meaning that a small fraction of the 7-step has an LR larger than 1 and a small fraction of

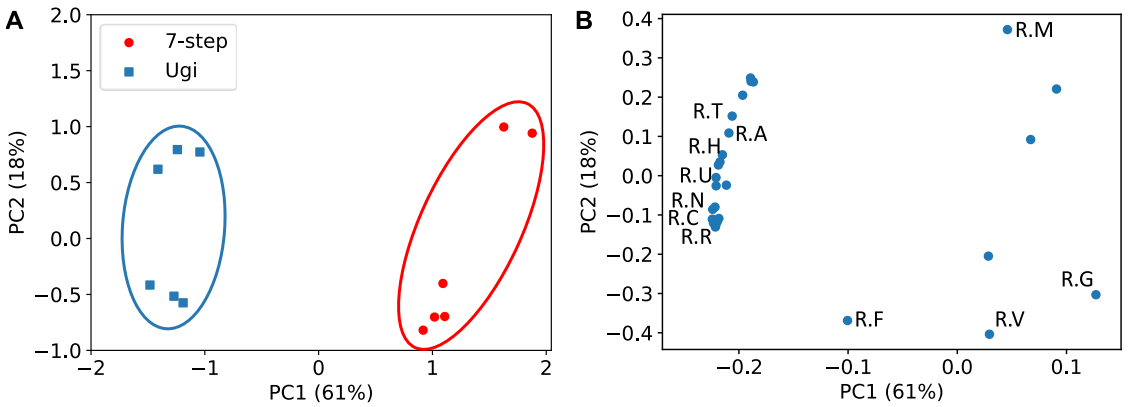


Fig. 4. A) PCA-score plot of post-metabolism samples of remifentanil synthesized by the 7-step (red circle) and Ugi (blue square) method. B) Corresponding PCA loading plot with highlighted impurities. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

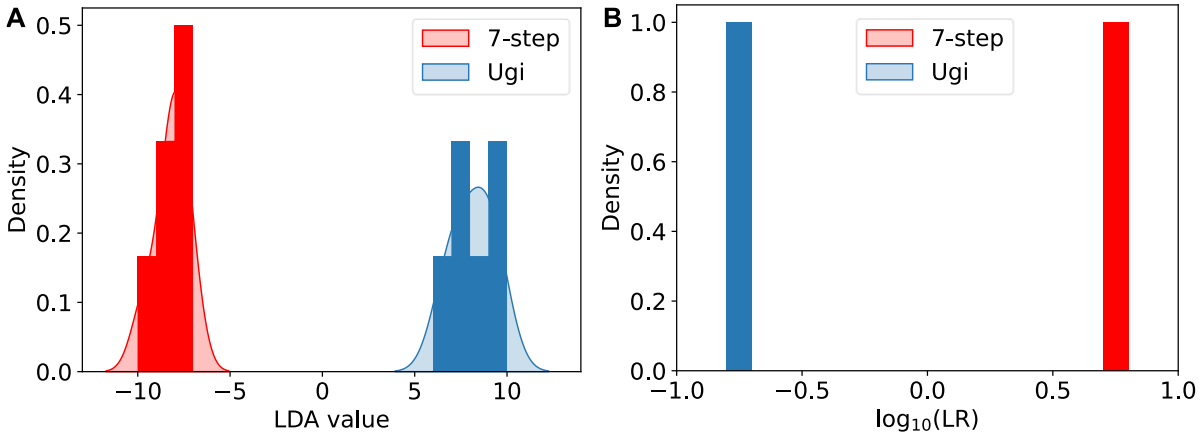


Fig. 5. A) LDA score plot B) Corrected distribution of log10 LRs with ELUB bounds, for remifentanil post-metabolism samples of 7-step synthesis (red) and Ugi method (blue), analyzed with LC-HRMS/MS. The bars show the frequency of the measurements, and the shaded curves represent the kernel density estimations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

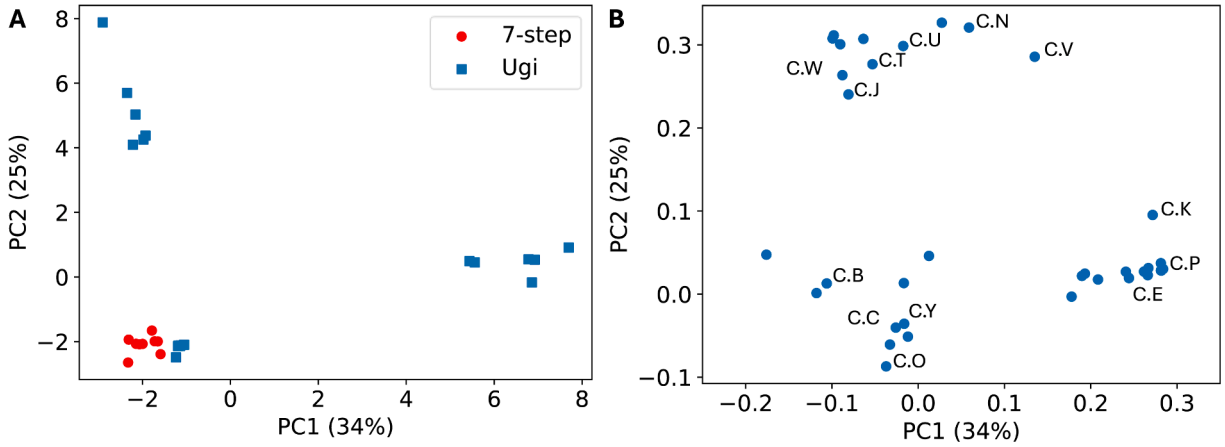


Fig. 6. PCA-score plot of post-metabolism samples of carfentanil synthesized by the 7-step (red circle) and Ugi (blue square) method. The datapoints in the top left are purified samples, and the other datapoints are batches of unpurified samples (left bottom) including two batches made by two different chemists (right bottom). B) Corresponding PCA loading plot with highlighted impurities. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the Ugi samples has an LR smaller than 1. Consequently, the rate of false negative results is 11 % for the 7-step method and 8.6 % for the Ugi synthesis method. No false positives and false negatives were found for the corrected ELUB LR distribution. Subsequently, the uncorrected LRs were in the range of 10^{-3} to 10^{45}. In comparison with the remifentanil results, the corrected ELUB LR values were slightly higher between 0.11 and 16. Section S6 of the Supplementary data presents the Tippett plots and pre-metabolism results. Extremely low error rates below 0.00013 %

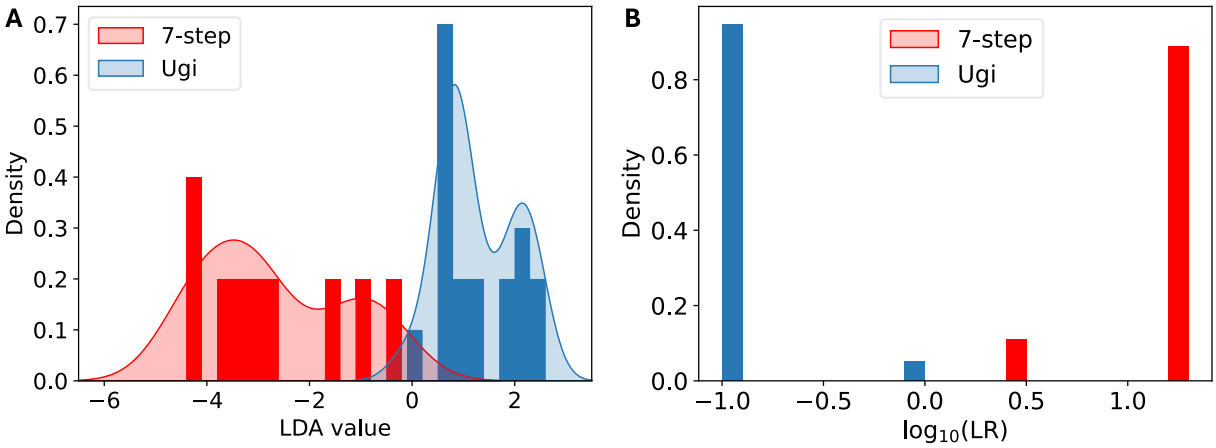


Fig. 7. A) LDA score plot B) Corrected distribution of log10 LRs with ELUB bounds, for carfentanil post-metabolism samples of 7-step synthesis (red) and Ugi method (blue), analyzed with LC-HRMS/MS. The bars show the frequency of the measurements, and the shaded curves represent the kernel density estimations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were found for the uncorrected pre-metabolism distribution of carfentanil and no misleading evidence was calculated for the ELUB LR plot. The LR distribution without correction was between 10^{-100} and 10^{150}. These values were reduced to 0.083 to 13 after applying ELUB bounds to prevent overestimation of the evidential value due to extrapolation. To conclude, these results indicate that if a positive LR is obtained for an unknown (biomedical) sample, the profile is more probable when the victim has been exposed to carfentanil produced according to the 7-step synthetic route (H_1), than when the victim has been exposed to carfentanil synthesized with the Ugi method (H_2).

3.5. Application in forensic casework

This section elaborates on the translation of the in-vitro impurity profiles to concentrations that might be encountered in actual forensic casework. Formin et al. reported carfentanil concentrations ranging from 2.7 – 10.4 ng/mL in urine samples of ten people and 0.2 – 9.3 ng/mL in blood samples of nine people that died from drug overdoses involving carfentanil [40]. In addition, Shanks and Behonick identified carfentanil in 262 postmortem toxicology casework samples in the range of 0.01 – 2 ng/mL [41]. It was hypothesized that carfentanil originated from contaminated street heroin. This was confirmed by several case studies where people were found dead after using heroin or other drugs. In most cases, the cause of death was confirmed as carfentanil intoxication or mixed drug intoxication. Concentrations associated with fatality from carfentanil were in the range of 0.01 – 0.6 ng/mL measured in femoral, iliac, cardiac, and subclavian blood [41]. In the current study, incubation using human liver microsomes was performed with a higher concentration of 1 μg/mL carfentanil. However, the measured concentrations after metabolism were between 0.09 – 5.9 ng/mL. This is in the same range as established in the carfentanil case studies.

Several other studies investigated life-threatening remifentanil concentrations. Riches et al. analyzed a urine sample of a casualty who had survived the Moscow theatre siege [26]. This siege was stopped by using aerosolized carfentanil and remifentanil. Traces of approximately 0.1 ng/mL norcarfentanil were found in urine five days after exposure, but it lacked traces of remifentanil (acid). Another study by Vanneste et al. discussed an acute remifentanil overdose where rapid intravenous injection of remifentanil occurred due to misuse of a syringe pump [42]. In this case, the calculated plasma concentration of 150 ng/mL remifentanil was considerably higher. In the present study, incubation was performed with a relatively high concentration of 100 μg/mL remifentanil. However, a much lower concentration below the calibration range of 5 ng/mL was qualitatively measured after metabolism. Therefore, the detected concentration may be in the range expected in real overdose fatalities, but further research is required to establish the detection limits and to confirm casework concentrations. Currently, there are no post-mortem toxicological reports published for sufentanil. Since the lethal dose is comparable to remifentanil, it is expected that similar trace concentrations will be found.

Additionally, benzylfentanyl has been encountered in biomedical casework samples. Adamowicz et al. identified benzylfentanyl in two fatal cases along with fentanyl, norfentanyl, and other drugs [43]. Concentrations of 67 – 110 ng/mL benzylfentanyl and 22 – 41 ng/mL norfentanyl were measured in postmortem blood samples. In the present study, a concentration of 100 μg/mL benzylfentanyl was applied. After incubation, a concentration of 38 ng/mL benzylfentanyl and 14 ng/mL norfentanyl was detected. These concentrations are even lower than encountered in the described casework samples. Therefore, the method is sensitive enough for potential impurity profiling in forensic casework.

Finally, it would be valuable if impurity profiling can be applied at the individual batch level. The current study mainly identified synthesis specific impurities. In this case, the impurity profile can be used to classify the synthesis method and gather intelligence information. The evidential value of the chemical attribution signature is then dependent on the rarity of the synthesis method. However, the present research also identified various markers for different batches. This suggests that it would be possible to link a sample to a specific laboratory. These variations can for example occur due to the application of different raw materials, concentrations, laboratory instruments, and purification methods. Additionally, the present study demonstrated that the same or related impurities were found in pre-metabolism batches compared to samples after metabolism. This indicates the potential of matching a profile of a blood sample of an exposed victim to a batch of intact material. It is therefore likely that post-metabolism impurity profiling can be applied to retrieve information about the synthesis route and to link a casework sample to a specific laboratory. This should however be confirmed by in-vivo testing or analyzing biomedical case work samples from victims.

4. Conclusions

The present study was designed to investigate the effect of human metabolism on the impurity profile of synthetic opioids carfentanil, remifentanil, sufentanil, and benzylfentanyl. Characteristic impurities were identified in pre- and post-metabolism samples with LC-HRMS/MS. It is important to note that the results should be interpreted with caution, since they were based on a limited sample set and the markers were only tentatively identified by LC-HRMS/MS without the use of validated reference standards. Nonetheless, this study has highlighted

12–24 markers for each fentanyl analogue. A major finding was the presence of precursors of the 7-step, Ugi, and Siegfried synthesis method in the post-metabolism samples, indicating that specific synthesis information is retained after metabolism. Additionally, N-dealkylation, (di)hydroxylation, and glucuronidation metabolites of precursors S5 (*m/z* 317.168) and S6 (*m/z* 331.184) were detected in the sufentanil samples. Also, metabolites of 4-ANBP, aniline, and N-phenylacetamide were identified in post-metabolism samples of benzylfentanyl, remifentanil, and carfentanil, respectively. The match criterion approach for remifentanil has shown that the impurities aniline and R.H ($C_{12}H_{14}N_2O_2$, *m/z* 219.113) are characteristic markers for the Ugi synthesis method and R.G ($C_{12}H_{27}NO_2$, *m/z* 218.212) is distinctive to the 7-step method. For carfentanil C.T ($C_{18}H_{26}N_2O_4$, *m/z* 335.197) was identified as an important marker for the Ugi method and C.Y ($C_{26}H_{49}NO_9$, *m/z* 520.349) for the 7-step method. Subsequently, another distinct impurity, C.P ($C_{18}H_{26}N_2O_3$, *m/z* 319.202), emerged in pre- and post-metabolism samples of carfentanil. The method was found to be sensitive enough for potential impurity profiling in forensic casework. LDA was applied to maximize discriminative power and KDE was used to express likelihood ratios for assigning unknown samples. The machine learning methods showed clear separation of the synthesis routes. Corrected likelihood ratios with ELUB bounds were in the range of 0.083 to 16. Although the small LR range reflects the limited dataset, the findings show the potential of constructing likelihood ratio models for post-metabolism impurity profiling to facilitate forensic investigations. Future studies should investigate the syntheses variations encountered in forensic casework. Also, in real-life, the purity of the fentanyl derivatives might be higher, especially in the case of materials produced by pharmaceutical companies. This will influence the number and level of impurities, making it more challenging to apply post-metabolism impurity profiling. In addition, further research needs to be done to validate the in-vitro model in real human biological samples. In conclusion, this work is consistent with the earlier observations of post-metabolism impurity profiling of fentanyl and demonstrates the potential of using biomedical samples for retrieving information about the production method of synthetic opioids after exposure.

CRediT authorship contribution statement

Daan Vangerven: Writing – original draft, Validation, Software, Methodology, Investigation, Formal analysis. **Mirjam de Bruin-Hoegée:** Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Conceptualization. **Fleur Kerstens:** Validation, Methodology, Investigation, Formal analysis. **Meike Kerklaan:** Validation, Methodology, Investigation, Formal analysis. **Rowdy P.T. Bross:** Methodology, Investigation. **Alex Fidder:** Methodology, Investigation. **Marcel J. van der Schans:** Writing – review & editing, Supervision. **Daan Noort:** Writing – original draft, Validation, Software, Methodology, Investigation, Formal analysis. **Arian C. van Asten:** Writing – review & editing, Supervision.

Declaration of competing interest

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.forc.2024.100587.

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