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Detection of fentanyl, synthetic opioids, and ketamine in hair specimens from purposive samples of American and Italian populations

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ABSTRACT

With the current crisis related to the diffusion of fentanyl and other novel opioids in several countries and populations, new and effective approaches are needed to better elucidate the phenomenon. In this context, hair testing offers a unique perspective in the investigation of drug consumption, producing useful information in terms of exposure to psychoactive substances. In this research, we applied targeted ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) analytical methods to detect novel synthetic and prescription opioids and other common controlled psychoactive drugs in the keratin matrix. A total of 120 hair samples were analyzed from the United States (US) and Italy, segmented when longer than 6 cm, and then analyzed. In the 60 samples (83 segments in total) analyzed from a purposive sample of data collected in [the US](#), fentanyl was detected in 14 cases (16.9%), with no detection of nitazens or brorphine. [We also detected](#) fentanyl metabolites, despropionyl-*p*-fluorofentanyl, and prescription opioids. In the 60 samples collected in Italy (91 segments in total), ketamine was the most prevalent compound detected (in 41 cases; [45.1%](#)), with ketamine demonstrating a strong correlation with detection of amphetamines and MDMA, likely due to co-use of these substances in recreational contexts. Several common drugs were also detected, but no exposure to fentanyl or its analogs were detected. Results of this retrospective exploration of drug use add to increasing evidence that hair testing can serve as a useful adjunct to epidemiology studies that seek to determine biologically confirmed use and exposure in high-risk populations.

Keywords: hair analysis; fentanyl; synthetic opioids; ketamine; [new psychoactive substances](#); [MDMA](#)

Highlights

- Results from real samples are crucial to understand the molecules used by at-risk populations
- In the US, fentanyl was detected in 16.9% of cases, with no detection of nitazens or buporphine
- In Italy, ketamine was the most prevalent compound detected, detected in 45.1% of hair segments
- Detection of ketamine was strongly correlated with detection of amphetamines and MDMA
- Hair analysis is effective in investigating the diffusion of new psychoactive drugs

1. Introduction

In the past decade, illicit opioid use has progressed from nonmedical use of legal analgesic drugs such as hydrocodone, oxycodone, and tramadol, to the diffusion of illicitly manufactured fentanyl and its analogs, often referred to as “fentologs” (1, 2). In addition, a new class of synthetic opioids referred to as nitazenes has been recently reported in several illegal drug markets (3). Many of these novel synthetic opioids (NSOs) are considered particularly risky due to their high potency and because they are often introduced into the market as cutting/adulterant agents of drugs such as heroin or simply as cheaper substitutes for other drugs (4, 5).

While heroin was the first drug to become frequently replaced or cut with NSO, these compounds began to appear to counterfeit pills representing common prescription drugs (e.g., oxycodone, alprazolam) and other illegal powder drugs such as cocaine (5-10), raising a major health concern for unaware users. In parallel, however, preference for fentanyl has increased among some populations, leaving doubt regarding whether fentanyl is more demand-led or supply-led (11). However, it is important to note that currently, the synthetic opioid crisis is centered in North America. In 2022, in the United States (US), there were 71,238 deaths linked to use of synthetic opioids such as fentanyl (12), and in 2021, there were at least 10,000 fentanyl seizures in the US which weighed over 10,000 kg in total (13). In Europe, however, among 12 countries providing seizure data to the European Union Early Warning System, in 2021, there were only 187 recorded fentanyl seizures (weighting 5.5 kg in total) (14). Heroin has largely been replaced by fentanyls in the US, but heroin is still the most common illicit opioid in Europe (14). In Italy, an average of 0.74 tons of heroin have been seized annually between 2011 and 2021 (14).

Despite differences in the opioid and other drug landscapes between the US and Europe, new and effective approaches are needed to monitor shifting drug-related phenomena. For example, in the US, in 2021, there was an increase to 24,538 deaths linked to cocaine use and 32,856 deaths linked to other psychostimulant (mainly methamphetamine) use (12), but the vast majority of such cases involve co-use of opioids (15). A greater understanding of co-use (or co-exposure) of illicit fentanyl,

opiods, and other psychoactive drugs can help adapt and improve existing interventions aimed to reduce overdose mortality, together with broad integrated public health strategies based on overdose education and prevention and to support the drugs debate (16-18).

In this context, hair analysis has proved to be an easy and effective tool to investigate the prevalence of use of psychoactive substances, since the keratin matrix allows for the detection of past drug exposure and for the investigation of the chronological profile of the exposure to one or multiple compounds. Furthermore, hair analysis is now based on multiclass methods for both well-known and emerging compounds, allowing for the investigation of different consumption patterns, including co-use of common drugs (including prescription opiods), as well as occasional vs. frequent NSO use or exposure (19-21). While several papers have described multianalyte screening methods capable of detecting NSOs (22), few have presented results from real samples (23-25). Polydrug use has generally been shown to be common based on the aforementioned studies, involving several psychoactive substances and not only heroin (26, 27). In general, fentanyl has been the most frequently detected compound among the class of fentologs (8, 19), suggesting that it is the most prevalent molecule while the less common analogs tend to be co-used with other drugs and are thus not consumed in isolation. Other typical matters of current discussion (in order to provide a definitive interpretation of positive vs. negative results) are: (i) the meaning of quantitative results, in terms of occasional and frequent use or exposure), and (ii) the identification of proper metabolites to discriminate direct exposure from potential external contamination.

In this paper, we present our ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) analysis of a subset of hair samples collected in the US and in Italy based on purposive sampling methods. All samples were screened for fentologs, prescription opiods, nitazens, bupropion, and other common controlled psychoactive drugs.

2. Materials and methods

2.1. Reagents and standards

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All chemicals, including methanol, formic acid and acetonitrile, were purchased from Sigma-Aldrich (Milan, Italy) while ultra-pure water was obtained using a Milli-Q® UF-Plus apparatus (Millipore, Bedford, MA, USA). The analytical standards of the target analytes and deuterated internal standards (norfentanyl-D5, fentanyl-D5 and oxycodone-D6) were purchased from LGC Promochem (Milan, Italy) and Sigma-Aldrich (Milan, Italy) (purity >99%, concentration between 0.1 mg/mL and 1 mg/mL), or kindly provided by the Italian National Early Warning System (provided at a concentration of 0.02 mg/mL). [The list of target analytes is presented in Table 1.](#) All stock standard solutions were prepared in methanol at 1 mg/mL and stored at -20°C until used. Working solution of 42 analytes (identified among the most common synthetic opioids and those recently observed by the warning systems) and internal standard solution were prepared at the final concentration of 1 µg/mL by dilution with methanol.

2.2. Sample collection and preparation

In this study, we focus on two purposive samples of adults—from the US and from Italy. Hair samples were collected in 2022 in the US (60 samples, from an ongoing rapid street reporting surveillance study being conducted throughout various US cities by the National Drug Early Warning System) (28) and in Italy (60 samples, from harm reduction services in Northern Italy), according to international guidelines (29). With regard to the 60 US samples, we focused on samples provided by participants who reported past-12 month use of heroin and/or fentanyl (n=18), 21 participants reporting past-12-month use of at least one novel psychoactive substance (NPS; who did not report heroin or fentanyl use; n=21), and a random sample of 21 participants who did not report heroin, fentanyl, or NPS use (n=21). In order to nullify any further risk related to data sharing and to safeguard the privacy of sample donors, in the US, all samples were collected anonymously. Italian samples were made anonymous by alphanumeric codes and used only in our laboratory. The risk of re-identification was also nullified. Furthermore, subjects provided informed consent to be tested for drug exposure. The study protocol for hair sample collection and testing for US samples was reviewed

and approved by the Institutional Review Board (IRB) of the University of Florida. The study protocol for hair sample collection in Italy was approved by the Bioethical Committee of the University of Turin.

All samples were analyzed up to a maximum of the proximal 12 cm, since the study aimed to explore the intake of drugs in the 12 months prior to collection (assuming a normal hair growth rate of 1 cm per month). When hair was ≤ 6 cm, it was analyzed in its entire length. When hair was longer than 6 cm (54 samples), two segments were prepared for analysis (with one representing roughly the past six months and the other representing roughly the previous 6-12 months). Therefore, a total of 174 separate segments was analyzed. The targeted screening for common drugs was performed using previously published and fully validated methods (30, 31). Existing procedures for novel opioids (32, 33) were adjusted to expand the panel of screened molecules. A partial validation was performed, aimed to verify sensitivity and specificity. The limits of detection are presented in Table S1, while data for trueness and precision at three different concentration levels are presented in Table S2.

All samples were treated with a procedure developed on-purpose for the keratin matrix. About 50 mg of hair was decontaminated by an initial wash with 1-mL dichloromethane followed by a second wash with 1-mL methanol, each one performed under 3 min stirring. The dried hair was pulverized using six steel balls stirring in a Precellys® homogenizer. The pulverized samples were extracted by keeping them immersed in 0.5-mL methanol added with 2.5 μ L of an internal standards mixture (final concentration of 0.01 ng/mg) at $+55 \pm 5^\circ\text{C}$ for 15 h. Lastly, the organic phase was collected and an aliquot of 3 μ L was directly injected into the UHPLC–MS–MS system. A calibration curve in the range 10-250 pg/mg was also prepared by spiking the proper quantities of analytical standards into a blank hair sample.

2.3 Instrumentation

UHPLC separation was performed with a Phenomenex Kinetex C18 column (100 \times 2.1 mm, 1.7 μ m) maintained at 45 $^\circ\text{C}$ on the SCIEX ExionLC™ AC system. The mobile phases consisted of

water (A) and acetonitrile (B), both with formic acid 5 mM. The LC flow rate was set at 0.5 mL/min and the mobile phase eluted under the following linear gradient conditions: (A:B, v:v) isocratic elution at 95:5 for 0.5 min, from 95:5 to 5:95 in 7.5 min, isocratic elution at 5:95 for 0.5 min and final re-equilibration for 1.5 min to the initial condition. The total run time was 10 min. All analyses were performed using a mass spectrometer equipped with a quadrupole trap SCIEX triple Quad™ 7500 mass spectrometer (Sciex, Darmstadt, Germany) system equipped with an OptiFlow Pro ion source with an analytical probe and E Lens. The ionization source was operated with electrospray ionization (ESI) in the positive mode. For each transition, compound-specific parameters such as collision energy (CE) were also optimized after infusion of the standard solution. A single acquisition method was created using the Scheduled MRM algorithm in SCIEX OS software 2.0. Three MRM transitions were monitored for each targeted analyte. The full list of the target analytes, the monitored transitions, and their instrumental parameter are reported in Table 1.

2.4 Statistical Analysis

We used descriptive statistics to describe the number of segments testing positive for various drugs in each country, and among positive cases we also described the range of levels of molecules detected. Within the Italian sample, we also computed Spearman correlations to determine the extent to which level of detection of each drug was correlated.

3. Results and discussion

Testing for NSO

In the 60 samples collected in the US (comprising of 83 segments), at least one opioid was detected in 16 segments (19.3%). Fentanyl was detected above the LOD (estimated at 5 pg/mg) in 14 segments (16.9%). The range of measured concentrations of fentanyl was extremely wide, ranging from 13 pg/mg through 7300 pg/mg, with a mean value of 1377 pg/mg and a median of 382 pg/mg.

Only 4 segments measured below 100 pg/mg (0.1 ng/mg). In eight segments, external contamination was excluded because the metabolite norfentanyl was also detected in the range 32 pg/mg – 2300 pg/mg, with a mean value of 809 pg/mg and a median of 209 pg/mg. Another promising marker of active fentanyl use, beta-hydroxyfentanyl (34), was detected in six cases, in the range 17 pg/mg - 1400 pg/mg. However, when beta-hydroxyfentanyl was present, norfentanyl was as well. Overall, the main metabolites were detected in the majority of hair samples testing positive for fentanyl supporting the possibility to ascertain active use. Acetylfentanyl, which is suggestive of clandestine production, was detected in 3 cases (range: 129 pg/mg – 265 pg/mg) and 4-ANPP, which is a precursor of fentanyl, in 7 cases (range: 23 pg/mg-2200 pg/mg), confirming that these two molecules are often present in hair samples from people exposed to fentanyl, as a by-product of either metabolism or synthesis of fentanyl. One further fentalog, despropionyl-p-fluorofentanyl, was detected in one sample at the concentration of 25 pg/mg, together with fentanyl at 2900 pg/mg. The sporadic occurrence of the other fentalogs has different possible explanations: (i) low prevalence within the populations assessed at the time of the sample collection, (ii) poor incorporation or low stability in the keratin matrix, and/or (iii) insufficient sensitivity of the analytical method in relation to the low effective dosage. A summary of results is presented in Table 2.

Three prescription opioids were detected in seven segments (8.4%), usually together with fentanyl. Only two segments (collected from the same subject) followed a different trend, with fentanyl below the LOD and hydrocodone measured at 37 pg/mg and 46 pg/mg, respectively. The trace level of fentanyl detected might indicate unintended exposure as an adulterant or contaminant if the drugs were obtained illegally. The nitazene compounds and buprenorphine, which appeared to have a significant presence in the NPS opioid market in 2019 and 2020 (3), were not detected. Although the number of samples analyzed in this study was relatively small, the non-detection of emerging opioids is coherent with the modern drug scenario, in which the typical life cycle of a new substance is generally short. Most new drugs appear to remain in circulation less than six months and

up to one year, but then rapidly decline, disappear, and then are replaced by other newly emerging synthetic substances (3).

Novel and prescription synthetic opioid identification was much less common in the 60 samples collected in Italy. In particular, fentalogs were never detected, while only five segments (5.5%) were positive for at least one compound such as hydrocodone, oxycodone, or tramadol. Two subjects were positive for all three prescription opioids. In one case, two segments were obtained from the same sample, showing the same trend of consumption (hydrocodone at 19 and 22 pg/mg, respectively).

Testing for common drugs

Samples were considered positive in accordance with international cut-offs for parent drugs and metabolites (35). In the group of samples from the US, cocaine was the most prevalent substance found in the samples, with 19 segments (22.9%) resulting above the cut-off for either cocaine or its metabolite benzoylecgonine (BZE). Cocaethylene was detected above 0.05 ng/mg only in five cases. The 6-acetylmorphine (6-MAM) as marker of heroin use was identified in five segments (6.0%), and all samples positive for 6-MAM also tested positive for BZE. While it is not possible to discriminate whether cocaine and heroin were taken simultaneously or in rapid sequence, the fact that the two substances were used in the same six months is remarkable. Use of cannabis-derived products was verified by the presence of Δ^9 -tetrahydrocannabinol (THC) in only eight segments (9.6%). It is noteworthy that the frequent use of amphetamine/methamphetamine/MDMA as a whole was observed in 26 segments (31.3%), of which 14 tested positive also to cocaine. Ketamine was detected in only one segment, in contradiction with the increasing trend recently reported, especially in the New York City area (36, 37).

An exhaustive comparison of patterns of drug use between the US and Italy based on the group of results hereby presented is not possible, nor is this the goal of our research. However, a striking difference emerges from the results obtained from the samples collected in Italy. Among 91

segments, a total of 41 (45.1%) tested positive for exposure ketamine in the range 245-8500 pg/mg (mean value 2324 pg/mg, median 1496 pg/mg). The large majority of samples positive for ketamine also tested positive for MDMA and/or cocaine, showing a trend of potential co-use of stimulating and dissociative substances. Overall, THC was still the most prevalent parent drug, with 73 positive segments (80.2%). The use of heroin, proven by the presence of 6-MAM, was identified in eight segments (8.8%).

Correlation matrices for the measured levels of common drugs in the Italian population of hair samples are presented in Figure 1. High correlation coefficients suggest that subjects who were more exposed to ketamine were more exposed to amphetamine, possibly due to a co-use of the substances in certain recreational contexts.

4. Conclusions

Hair analysis can help to retrospectively explore trends in drug use, and incorporating hair testing into epidemiology studies or surveillance studies can provide opportunity for relatively rapid dissemination of results (including public alerts) to both the scientific community and populations at risk.

_____ In this analysis focusing on hair samples collected in the US and in Italy, we tested for use or exposure to fentologs, prescription opioids, and more common controlled drugs including ketamine. Results suggest that currently fentologs continue to be a US (or North American) phenomenon, with no detected cases in Italy despite high prevalence of detection of other drugs within this country. However, we checkedinvestigated samples from two different populations within these two countries, and results should not be directly used to indicate prevalence of drug use, as we used purposive sampling. As such, results are not generalizable to US or Italian populations, but rather present a snapshot of drug use within select populations in each country. Indeed, all cases of synthetic opioid detection were in the US, but we focused on a sample in which many participants reported recent synthetic opioid use. Most use of common party drugs such as ketamine and MDMA were detected

in the Italian sample, but we must keep in mind that these were individuals receiving harm reduction services associated with nightlife. People who attend nightclubs in particular tend to report higher prevalence of use of such drugs than the general population (38-40). Prevalence of past-year ketamine use among young adults is estimated to be <1% in both the US and in Europe (14, 41), although seizures of the drug appear to be increasing at a similar rate (14, 42). While prevalence of past-year heroin use is estimated to be <1% in both the US and Europe (14, 43), in the US, synthetic opioids such as fentanyl analogs indeed are more available and have been involved in hundreds of thousands of deaths in recent years (13, 44). As such, it is important to note both where biological specimens are collected but also the populations from which they are obtained. This is because results will vary in particular across high-risk populations (e.g., nightclub attendees, people who utilize drug checking services) and the general population.

Thanks to the longer detection window of hair (in comparison to much shorter detection windows provided by urine, saliva, and blood), drug exposures occurring 1-2 weeks up through a year before hair collection can provide retrospective results to inform scientists and public health practitioners about the diffusion of drugs in their countries. Hair analysis results based on real hair samples can provide information regarding both intentional and unintentional exposure to NPS/NSO, both with and without use of common controlled drugs. As such, hair testing can serve as an addition to epidemiology studies that seek to incorporate biological testing with survey research. The combination of surveys and hair testing can thus be used to monitor drug exposure in a more effective manner than using surveys or biological testing alone.

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Table 1: List (ordered by RT) of the monitored transitions, their instrumental parameters and the related internal standard for the screened compounds.

<u>Compound</u>	<u>Retention time, min</u>	<u>Precursor mass Q1 m/z</u>	<u>Fragments mass Q3 m/z</u>	<u>CE (V)</u>	<u>EP (V)</u>	<u>Internal Standard</u>
<u>Acetyl norfentanyl</u>	<u>2.10</u>	<u>219.1</u>	<u>84.1</u> <u>55.1</u> <u>56.1</u>	<u>23</u> <u>48</u> <u>42</u>	<u>8</u>	<u>Fentanyl-D5</u>
<u>Methoxyacetyl norfentanyl</u>	<u>2.10</u>	<u>249.1</u>	<u>84.1</u> <u>55.1</u> <u>56</u>	<u>22</u> <u>55</u> <u>40</u>	<u>8</u>	<u>Fentanyl-D5</u>
<u>Oxycodone</u>	<u>2.10</u>	<u>316.0</u>	<u>298.1</u> <u>241.1</u> <u>256.1</u>	<u>25</u> <u>38</u> <u>34</u>	<u>8</u>	<u>Oxycodone-D6</u>
<u>Hydrocodone</u>	<u>2.20</u>	<u>300.0</u>	<u>199.1</u> <u>171.1</u> <u>128.0</u>	<u>42</u> <u>51</u> <u>74</u>	<u>8</u>	<u>Fentanyl-D5</u>
<u>Norfentanyl</u>	<u>2.60</u>	<u>233.0</u>	<u>84.1</u> <u>150.1</u> <u>55.0</u>	<u>24</u> <u>22</u> <u>50</u>	<u>8</u>	<u>Norfentanyl-D5</u>
<u>Metodesnitazene</u>	<u>2.60</u>	<u>338.1</u>	<u>100.0</u> <u>72.0</u> <u>121.0</u>	<u>23</u> <u>53</u> <u>50</u>	<u>8</u>	<u>Fentanyl-D5</u>
<u>3-methyl Norfentanyl</u>	<u>2.70</u>	<u>247.1</u>	<u>98.1</u> <u>150.1</u> <u>69.0</u>	<u>23</u> <u>26</u> <u>42</u>	<u>8</u>	<u>Fentanyl-D5</u>
<u>Furanyl Norfentanyl</u>	<u>2.70</u>	<u>271.2</u>	<u>84</u> <u>56.1</u> <u>55</u>	<u>20</u> <u>41</u> <u>54</u>	<u>8</u>	<u>Fentanyl-D5</u>
<u>Tramadol</u>	<u>2.80</u>	<u>264.1</u>	<u>58.1</u> <u>246.1</u>	<u>46</u> <u>15</u>	<u>8</u>	<u>Fentanyl-D5</u>
<u>Butiryl Norfentanyl</u>	<u>3.00</u>	<u>247.2</u>	<u>84.0</u> <u>177.2</u> <u>55.0</u>	<u>24</u> <u>21</u> <u>55</u>	<u>8</u>	<u>Fentanyl-D5</u>
<u>Etodesnitazene</u>	<u>3.00</u>	<u>352.1</u>	<u>100.1</u> <u>71.9</u> <u>107.1</u>	<u>26</u> <u>57</u> <u>60</u>	<u>8</u>	<u>Fentanyl-D5</u>
<u>Remifentanyl</u>	<u>3.10</u>	<u>377.1</u>	<u>317.2</u> <u>228.0</u> <u>116.1</u>	<u>22</u> <u>27</u> <u>37</u>	<u>8</u>	<u>Fentanyl-D5</u>
<u>Butyrylfentanyl carboxy metabolite</u>	<u>3.10</u>	<u>381.1</u>	<u>188.2</u> <u>105.1</u> <u>260.1</u>	<u>34</u> <u>56</u> <u>34</u>	<u>8</u>	<u>Fentanyl-D5</u>

OH-thioentanyl	3.10	359.1	192.1 146.1 111.0	32 32 50	8	Fentanyl-D5
Valeryl p-fluoro fentanyl	3.20	395.1	188.2 105.0 274.1	32 57 33	8	Fentanyl-D5
Acetylfentanyl	3.20	323.0	188.2 105.0 103.0	38 38 86	8	Fentanyl-D5
Ocfentanyl	3.20	371.1	105.1 188.2 134.0	50 31 38	8	Fentanyl-D5
beta-OH-fentanyl	3.30	353.2	186.1 204.2 335.2	32 28 26	8	Fentanyl-D5
4-ANPP	3.40	281.0	188.2 105.0 103.0	24 41 63	8	Fentanyl-D5
Alfentanyl	3.50	417.0	268.3 197.2 165.0	24 35 47	8	Fentanyl-D5
Acrylfentanyl	3.50	335.1	188.2 105.0 132.1	30 50 42	8	Fentanyl-D5
Despropionyl-p- fluorofentanyl	3.50	299.2	188.1 105.0 134.0	24 39 32	8	Fentanyl-D5
Flunitazene	3.50	371.1	100.1 109.1 72.1	33 65 58	8	Fentanyl-D5
Fentanyl	3.50	337.1	188.2 105.0 132.1	32 49 42	8	Fentanyl-D5
Metonitazene	3.50	383.0	100.0 72.1 121.0	26 58 38	8	Fentanyl-D5
U-47700	3.50	328.9	204.1 286.1 206.1	36 24 34	8	Fentanyl-D5
4-methylfentanyl	3.60	351.1	91 202.1 119.1	51 30 35	8	Fentanyl-D5
AH-7921	3.60	329.0	173.0 284.1 286.1	40 23 24	8	Fentanyl-D5
Furanilfentanyl	3.60	375.0	188.2 105.0 103.0	28 52 82	8	Fentanyl-D5

<u>Brorphine</u>	<u>3.70</u>	<u>402.0</u>	<u>218.2</u> <u>104.1</u> <u>218.2</u>	<u>29</u> <u>63</u> <u>35</u>	<u>8</u>	<u>Fentanyl-D5</u>
<u>Carfentanyl</u>	<u>3.70</u>	<u>395.0</u>	<u>335.2</u> <u>246.1</u> <u>113.0</u>	<u>25</u> <u>34</u> <u>34</u>	<u>8</u>	<u>Fentanyl-D5</u>
<u>Cyclopropylfentanyl</u>	<u>3.70</u>	<u>349.1</u>	<u>188.1</u> <u>105.0</u> <u>132.0</u>	<u>32</u> <u>51</u> <u>40</u>	<u>8</u>	<u>Fentanyl-D5</u>
<u>N-pyrrolidino etonitazene</u>	<u>3.70</u>	<u>395.0</u>	<u>98.0</u> <u>107.0</u> <u>56.0</u>	<u>27</u> <u>70</u> <u>82</u>	<u>8</u>	<u>Fentanyl-D5</u>
<u>Isotonitazene</u>	<u>3.70</u>	<u>411.2</u>	<u>100.0</u> <u>106.9</u> <u>72.0</u>	<u>20</u> <u>52</u> <u>42</u>	<u>8</u>	<u>Fentanyl-D5</u>
<u>Butyrylfentanyl</u>	<u>3.80</u>	<u>351.2</u>	<u>188.2</u> <u>105.1</u> <u>230.2</u>	<u>31</u> <u>49</u> <u>31</u>	<u>8</u>	<u>Fentanyl-D5</u>
<u>Phenyl fentanyl</u>	<u>3.80</u>	<u>385.2</u>	<u>188.2</u> <u>105</u> <u>134.2</u>	<u>29</u> <u>51</u> <u>36</u>	<u>8</u>	<u>Fentanyl-D5</u>
<u>Sufentanyl</u>	<u>3.90</u>	<u>387.0</u>	<u>238.1</u> <u>355.1</u> <u>111.0</u>	<u>26</u> <u>26</u> <u>46</u>	<u>8</u>	<u>Fentanyl-D5</u>
<u>4-F-butylfentanyl</u>	<u>3.90</u>	<u>369.1</u>	<u>188.1</u> <u>105.0</u> <u>248.1</u>	<u>33</u> <u>55</u> <u>33</u>	<u>8</u>	<u>Fentanyl-D5</u>
<u>Phenylacetyl fentanyl</u>	<u>4.10</u>	<u>399.2</u>	<u>105</u> <u>188.2</u> <u>134.1</u>	<u>55</u> <u>32</u> <u>39</u>	<u>8</u>	<u>Fentanyl-D5</u>
<u>MT-45</u>	<u>4.20</u>	<u>349.1</u>	<u>181.1</u> <u>166.2</u> <u>169.2</u>	<u>36</u> <u>46</u> <u>25</u>	<u>8</u>	<u>Fentanyl-D5</u>
<u>beta-phenyl Fentanyl</u>	<u>4.30</u>	<u>413.2</u>	<u>188.2</u> <u>105.0</u> <u>292.1</u>	<u>35</u> <u>55</u> <u>37</u>	<u>8</u>	<u>Fentanyl-D5</u>
<u>Butonitazene</u>	<u>4.4</u>	<u>425.2</u>	<u>100.1</u> <u>72.0</u> <u>107.0</u>	<u>31</u> <u>67</u> <u>75</u>	<u>8</u>	<u>Fentanyl-D5</u>

Table 2 Summary of results obtained from 60 real hair samples collected in the U.S. All concentrations are in pg/mg. Two segments from the same sample are referred to as *a* and *b*

<u>Sample</u>	<u>Fentanyl</u>	<u>Norfentanyl</u>	<u>4-ANPP</u>	<u>β-OH-fentanyl</u>	<u>Other fentalogs</u>	<u>Prescription opioids</u>
<u>1</u>	<u>90</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>
<u>2</u>	<u>384</u>	<u>43</u>	<u>29</u>	<u>71</u>	<u>-</u>	<u>-</u>
<u>3</u>	<u>501</u>	<u>69</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>
<u>4a</u>	<u>2900</u>	<u>1700</u>	<u>747</u>	<u>1400</u>	<u>Acetyl fentanyl 157</u> <u>Despropionyl p-fluorofentanyl 25</u>	<u>Tramadol</u> <u>1400</u>
<u>4b</u>	<u>2900</u>	<u>2000</u>	<u>737</u>	<u>1300</u>	<u>Acetyl fentanyl 129</u>	<u>Tramadol</u> <u>1200</u>
<u>5a</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>Hydrocodone</u> <u>37</u>
<u>5b</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>Hydrocodone</u> <u>46</u>
<u>6</u>	<u>15</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>Hydrocodone</u> <u>26</u> <u>Tramadol 13</u> <u>Oxycodone</u> <u>128</u>
<u>7</u>	<u>13</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>
<u>8</u>	<u>103</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>
<u>9</u>	<u>331</u>	<u>32</u>	<u>23</u>	<u>-</u>	<u>-</u>	<u>-</u>
<u>10</u>	<u>1800</u>	<u>150</u>	<u>73</u>	<u>60</u>	<u>-</u>	<u>-</u>
<u>11</u>	<u>143</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>
<u>12a</u>	<u>2800</u>	<u>268</u>	<u>223</u>	<u>17</u>	<u>-</u>	<u>Hydrocodone</u> <u>78</u> <u>Tramadol 25</u> <u>Tramadol</u> <u>1000</u>
<u>12b</u>	<u>7300</u>	<u>2300</u>	<u>2200</u>	<u>980</u>	<u>Acetyl fentanyl 265</u>	<u>-</u>
<u>13</u>	<u>61</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>	<u>-</u>

Table 1: List of the monitored transitions, their instrumental parameters and the related internal standard for the screened compounds.

Compound	Retention time, min	Precursor mass Q1 m/z	Fragments mass Q3 m/z	CE (V)	EP (V)	Internal Standard
4-ANPP	3.40	281.0	188.2 105.0 103.0	24 41 63	8	Fentanyl-D5
Acetylfentanyl	3.20	323.0	188.2 105.0 103.0	38 38 86	8	Fentanyl-D5
Carfentanyl	3.70	395.0	335.2 246.1 113.0	25 34 34	8	Fentanyl-D5
Fentanyl	3.50	337.1	188.2 105.0 132.1	32 49 42	8	Fentanyl-D5
Furanilfentanyl	3.60	375.0	188.2 105.0 103.0	28 52 82	8	Fentanyl-D5
Hydrocodone	2.20	300.0	199.1 171.1 128.0	42 51 74	8	Fentanyl-D5
Norfentanyl	2.60	233.0	84.1 150.1 55.0	24 22 50	8	Norfentanyl-D5
Remifentanyl	3.10	377.1	317.2 228.0 116.1	22 27 37	8	Fentanyl-D5
Tramadol	2.80	264.1	58.1 246.1	46 15	8	Fentanyl-D5
Alfentanyl	3.50	417.0	268.3 197.2 165.0	24 35 47	8	Fentanyl-D5
Oxycodone	2.10	316.0	298.1 241.1 256.1	25 38 34	8	Oxycodone-D6
Sufentanyl	3.90	387.0	238.1 355.1 111.0	26 26 46	8	Fentanyl-D5
U-47700	3.5	328.9	204.1 286.1 206.1	36 24 34	8	Fentanyl-D5
4-F-butylfentanyl	3.90	369.1	188.1 105.0	33 55	8	Fentanyl-D5

			248.1	33		
4-methylfentanyl	3.60	351.1	91 202.1 119.1	51 30 35	8	Fentanyl-D5
Acrylfentanyl	3.50	335.1	188.2 105.0 132.1	30 50 42	8	Fentanyl-D5
AH-7921	3.60	329.0	173.0 284.1 286.1	40 23 24	8	Fentanyl-D5
Butyrylfentanyl	3.80	351.2	188.2 105.1 230.2	31 49 31	8	Fentanyl-D5
Cyclopropylfentanyl	3.70	349.1	188.1 105.0 132.0	32 51 40	8	Fentanyl-D5
MT-45	4.20	349.1	181.1 166.2 169.2	36 46 25	8	Fentanyl-D5
Ocfentanyl	3.20	371.1	105.1 188.2 134.0	50 31 38	8	Fentanyl-D5
3-methyl Norfentanyl	2.70	247.1	98.1 150.1 69.0	23 26 42	8	Fentanyl-D5
Acetyl-norfentanyl	2.10	219.1	84.1 55.1 56.1	23 48 42	8	Fentanyl-D5
Butyrylfentanyl carboxy-metabolite	3.10	381.1	188.2 105.1 260.1	34 56 34	8	Fentanyl-D5
Despropionyl-p- fluorofentanyl	3.5	299.2	188.1 105.0 134.0	24 39 32	8	Fentanyl-D5
Valeryl p-fluoro fentanyl	3.20	395.1	188.2 105.0 274.1	32 57 33	8	Fentanyl-D5
beta-OH-fentanyl	3.30	353.2	186.1 204.2 335.2	32 28 26	8	Fentanyl-D5
beta-phenyl Fentanyl	4.30	413.2	188.2 105.0 292.1	35 55 37	8	Fentanyl-D5
Butyryl Norfentanyl	3.00	247.2	84.0 177.2 55.0	24 21 55	8	Fentanyl-D5
Furanyl Norfentanyl	2.70	271.2	84	20	8	Fentanyl-D5

			56.1 55	41 54		
Methoxyacetyl norfentanyl	2.10	249.1	84.1 55.1 56	22 55 40	8	Fentanyl-D5
OH-thioentanyl	3.10	359.1	192.1 146.1 111.0	32 32 50	8	Fentanyl-D5
Phenylacetyl fentanyl	4.10	399.2	105 188.2 134.1	55 32 39	8	Fentanyl-D5
Phenyl fentanyl	3.80	385.2	188.2 105 134.2	29 51 36	8	Fentanyl-D5
Brorphine	3.70	402.0	218.2 104.1 218.2	29 63 35	8	Fentanyl-D5
Butonitazene	4.4	425.2	100.1 72.0 107.0	31 67 75	8	Fentanyl-D5
Etodesnitazene	3.00	352.1	100.1 71.9 107.1	26 57 60	8	Fentanyl-D5
Flunitazene	3.50	371.1	100.1 109.1 72.1	33 65 58	8	Fentanyl-D5
Metodesnitazene	2.60	338.1	100.0 72.0 121.0	23 53 50	8	Fentanyl-D5
Metonitazene	3.50	383.0	100.0 72.1 121.0	26 58 38	8	Fentanyl-D5
N-pyrrolidino etonitazene	3.70	395.0	98.0 107.0 56.0	27 70 82	8	Fentanyl-D5
Isotonitazene	3.68	411.2	100.0 106.9 72.0	20 52 42	8	Fentanyl-D5

Table 2 Summary of results obtained from 60 real hair samples collected in the U.S. All concentrations are in pg/mg. Two segments from the same sample are referred to as *a* and *b*.

Sample	Fentanyl	Norfentanyl	4-ANPP	β -OH-fentanyl	Other fentalogs	Prescription opioids
1	90	-	-	-	-	-
2	384	43	29	71	-	-
3	501	69	-	-	-	-
4a	2900	1700	747	1400	Acetyl-fentanyl 157 Despropionyl p-fluorofentanyl 25	Tramadol 1400
4b	2900	2000	737	1300	Acetyl-fentanyl 129	Tramadol 1200
5a	-	-	-	-	-	Hydrocodone 37
5b	-	-	-	-	-	Hydrocodone 46
6	15	-	-	-	-	Hydrocodone 26 Tramadol 13 Oxycodone 128
7	13	-	-	-	-	-
8	103	-	-	-	-	-
9	331	32	23	-	-	-
10	1800	150	73	60	-	-
11	143	-	-	-	-	-
12a	2800	268	223	17	-	Hydrocodone 78 Tramadol 25
12b	7300	2300	2200	980	Acetyl-fentanyl 265	Tramadol 1000
13	61	-	-	-	-	-

Legend Figure 1

Correlation matrices for the measured levels of common drugs of abuse in the Italian population

For Peer Review

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Table 1: List (ordered by RT) of the monitored transitions, their instrumental parameters and the related internal standard for the screened compounds.

Compound	Retention time, min	Precursor mass Q1 m/z	Fragments mass Q3 m/z	CE (V)	EP (V)	Internal Standard
Acetyl norfentanyl	2.10	219.1	84.1	23	8	Fentanyl-D5
			55.1	48		
			56.1	42		
Methoxyacetyl norfentanyl	2.10	249.1	84.1	22	8	Fentanyl-D5
			55.1	55		
			56	40		
Oxycodone	2.10	316.0	298.1	25	8	Oxycodone-D6
			241.1	38		
			256.1	34		
Hydrocodone	2.20	300.0	199.1	42	8	Fentanyl-D5
			171.1	51		
			128.0	74		
Norfentanyl	2.60	233.0	84.1	24	8	Norfentanyl-D5
			150.1	22		
			55.0	50		
Metodesnitazene	2.60	338.1	100.0	23	8	Fentanyl-D5
			72.0	53		
			121.0	50		
3-methyl Norfentanyl	2.70	247.1	98.1	23	8	Fentanyl-D5
			150.1	26		
			69.0	42		
Furanyl Norfentanyl	2.70	271.2	84	20	8	Fentanyl-D5
			56.1	41		
			55	54		
Tramadol	2.80	264.1	58.1	46	8	Fentanyl-D5
			246.1	15		
Butyryl Norfentanyl	3.00	247.2	84.0	24		Fentanyl-D5

			177.2	21	8	
			55.0	55		
Etodesnitazene	3.00	352.1	100.1	26		Fentanyl-D5
			71.9	57	8	
			107.1	60		
Remifentanyl	3.10	377.1	317.2	22		Fentanyl-D5
			228.0	27	8	
			116.1	37		
Butyrylfentanyl carboxy metabolite	3.10	381.1	188.2	34		Fentanyl-D5
			105.1	56	8	
			260.1	34		
OH-thioentanyl	3.10	359.1	192.1	32		Fentanyl-D5
			146.1	32	8	
			111.0	50		
Valeryl p-fluoro fentanyl	3.20	395.1	188.2	32		Fentanyl-D5
			105.0	57	8	
			274.1	33		
Acetylfentanyl	3.20	323.0	188.2	38		Fentanyl-D5
			105.0	38	8	
			103.0	86		
Ocfentanyl	3.20	371.1	105.1	50		Fentanyl-D5
			188.2	31	8	
			134.0	38		
beta-OH-fentanyl	3.30	353.2	186.1	32		Fentanyl-D5
			204.2	28	8	
			335.2	26		
4-ANPP	3.40	281.0	188.2	24		Fentanyl-D5
			105.0	41	8	
			103.0	63		
Alfentanyl	3.50	417.0	268.3	24		Fentanyl-D5
			197.2	35	8	
			165.0	47		

Acrylfentanyl	3.50	335.1	188.2 105.0 132.1	30 50 42	8	Fentanyl-D5
Despropionyl-p-fluorofentanyl	3.50	299.2	188.1 105.0 134.0	24 39 32	8	Fentanyl-D5
Flunitazene	3.50	371.1	100.1 109.1 72.1	33 65 58	8	Fentanyl-D5
Fentanyl	3.50	337.1	188.2 105.0 132.1	32 49 42	8	Fentanyl-D5
Metonitazene	3.50	383.0	100.0 72.1 121.0	26 58 38	8	Fentanyl-D5
U-47700	3.50	328.9	204.1 286.1 206.1	36 24 34	8	Fentanyl-D5
4-methylfentanyl	3.60	351.1	91 202.1 119.1	51 30 35	8	Fentanyl-D5
AH-7921	3.60	329.0	173.0 284.1 286.1	40 23 24	8	Fentanyl-D5
Furanilfentanyl	3.60	375.0	188.2 105.0 103.0	28 52 82	8	Fentanyl-D5
Brorphine	3.70	402.0	218.2 104.1 218.2	29 63 35	8	Fentanyl-D5
Carfentanyl	3.70	395.0	335.2 246.1	25 34	8	Fentanyl-D5

			113.0	34		
Cyclopropylfentanyl	3.70	349.1	188.1 105.0 132.0	32 51 40	8	Fentanyl-D5
N-pyrrolidino etonitazene	3.70	395.0	98.0 107.0 56.0	27 70 82	8	Fentanyl-D5
Isotonitazene	3.70	411.2	100.0 106.9 72.0	20 52 42	8	Fentanyl-D5
Butyrylfentanyl	3.80	351.2	188.2 105.1 230.2	31 49 31	8	Fentanyl-D5
Phenyl fentanyl	3.80	385.2	188.2 105 134.2	29 51 36	8	Fentanyl-D5
Sufentanyl	3.90	387.0	238.1 355.1 111.0	26 26 46	8	Fentanyl-D5
4-F-butylfentanyl	3.90	369.1	188.1 105.0 248.1	33 55 33	8	Fentanyl-D5
Phenylacetyl fentanyl	4.10	399.2	105 188.2 134.1	55 32 39	8	Fentanyl-D5
MT-45	4.20	349.1	181.1 166.2 169.2	36 46 25	8	Fentanyl-D5
beta-phenyl Fentanyl	4.30	413.2	188.2 105.0 292.1	35 55 37	8	Fentanyl-D5

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Butonitazene	4.4	425.2	100.1 72.0 107.0	31 67 75	8	Fentanyl-D5
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Table 2 Summary of results obtained from 60 real hair samples collected in the U.S. All concentrations are in pg/mg. Two segments from the same sample are referred to as *a* and *b*

Sample	Fentanyl	Norfentanyl	4-ANPP	β -OH-fentanyl	Other fentalogs	Prescription opioids
1	90	-	-	-	-	-
2	384	43	29	71	-	-
3	501	69	-	-	-	-
4a	2900	1700	747	1400	Acetyl fentanyl 157 Despropionyl p-fluorofentanyl 25	Tramadol 1400
4b	2900	2000	737	1300	Acetyl fentanyl 129	Tramadol 1200
5a	-	-	-	-	-	Hydrocodone 37
5b	-	-	-	-	-	Hydrocodone 46
						Hydrocodone 26
6	15	-	-	-	-	Tramadol 13 Oxycodone 128
7	13	-	-	-	-	-
8	103	-	-	-	-	-
9	331	32	23	-	-	-
10	1800	150	73	60	-	-
11	143	-	-	-	-	-
12a	2800	268	223	17	-	Hydrocodone 78 Tramadol 25
12b	7300	2300	2200	980	Acetyl fentanyl 265	Tramadol 1000
13	61	-	-	-	-	-

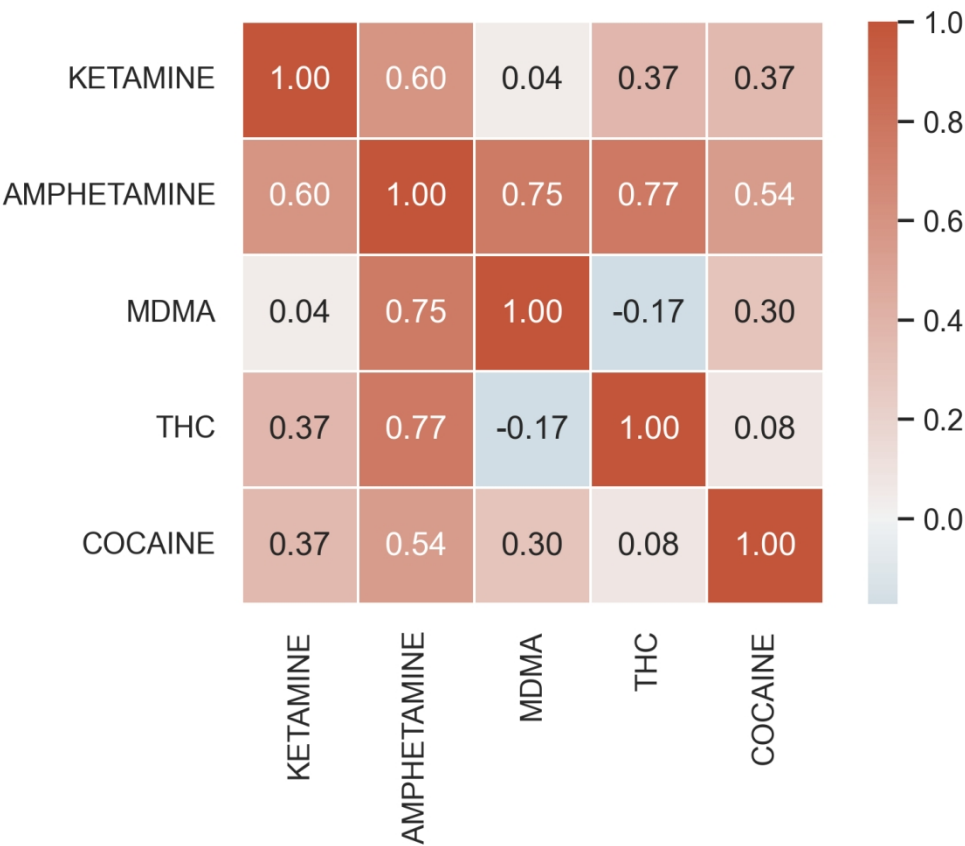


Figure 1

131x114mm (300 x 300 DPI)

Table S1: Limits of Detection (LOD) for the screened compounds. LOD were calculated from five injections of the lowest point of calibration curve.

Compound	LOD (pg/mg)
4-ANPP	1.7
Acetylfentanyl	2.8
Carfentanyl	2.2
Fentanyl	3.7
Furanilfentanyl	1.6
Hydrocodone	6.2
Norfentanyl	3.3
Remifentanyl	0.6
Tramadol	4.3
Alfentanyl	0.8
Oxycodone	1.7
Sufentanyl	3.5
U-47700	3.6
4-F-butylfentanyl	2.2
4-methylfentanyl	1.0
Acrylfentanyl	5.1

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AH-7921	3.5
Butyrylfentanyl	1.8
Ciclopropylfentanyl	2.8
MT-45	1.5
Ocfentanyl	1.6
3-methyl Norfentanyl	1.2
Acetyl norfentanyl	1.7
Butyrylfentanyl carboxy metabolite	1.9
Despropionyl-p- fluorofentanyl	3.1
Valeryl p-fluoro fentanyl	3.5
Beta-OH-fentanyl	0.8
Beta-phenyl Fentanyl	1.2
Butiryl Norfentanyl	1.7
Furanyl Norfentanyl	1.2
Methoxyacetyl norfentanyl	5.5
OH-ThioFentanyl	1.0
Phenylacetyl fentanyl	5.0

Phenyl fentanyl	1.8
Brorphine	0.7
Butonitazene	3.0
Etodesnitazene	5.4
Flunitazene	1.6
Metodesnitazene	8.0
Metonitazene	3.1
N-pyrrolidino etonitazene	4.1
Isotonitazene	5.6

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Table S2: Precision and trueness values at three different concentrations. A partial validation was performed for the compounds which were detected in real samples

Compound	Concentration (pg/mg)	Precision (CV%)	Trueness (BIAS%)
Fentanyl	10	14%	11%
	50	15%	-4%
	200	18%	-1%
Norfentanyl	10	5%	-20%
	50	6%	8%
	200	7%	0.1%
4-ANPP	10	18%	14%
	50	17%	2%
	200	12%	5%
β-OH-fentanyl	10	19%	15%
	50	20%	-8%
	200	20%	-1%
Acetyl fentanyl	10	5%	-14%
	50	8%	14%
	200	12%	-4%
Despropionyl p-fluorofentanyl	10	17%	18%
	50	19%	-17%
	200	17%	-0.2%
Tramadol	10	12%	-18%
	50	15%	9%
	200	10%	-1%
Hydrocodone	10	20%	-20%
	50	18%	16%
	200	19%	-2%
Oxycodone	10	14%	-12%
	50	7%	-2%
	200	15%	-1%

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