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**Toward the Interpretation of Positive Testing for Fentanyl and Its Analogs in Real Hair Samples:  
Preliminary Considerations**

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**Toward the interpretation of positive testing for fentanyl  
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3 **Toward the interpretation of positive testing for fentanyl and its analogs in real hair samples:**  
4 **preliminary considerations**  
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27 **Abstract**  
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30 The detection of NPS in hair has become extensively researched in recent years. Although most  
31 NPS fall into the classes of synthetic cannabinoids and designer cathinones, novel synthetic opioids  
32 (NSO) have appeared with increasing frequency in the illicit drug supply. While the detection of  
33 NSO in hair is now well-documented, interpretation of results presents several controversial issues,  
34 as is quite common in hair analysis. In this study, a UHPLC-MS/MS method able to detect 13  
35 synthetic opioids (including fentanyl analogs) and metabolites in hair was applied to 293 real  
36 samples. Samples were collected in the United States between November 2016 and August 2018  
37 from subjects who had reported heroin use in the past year or had already tested positive to hair  
38 testing for common opiates. The range, mean and median concentrations were calculated for each  
39 analyte, in order to draw a preliminary direction for a possible cut-off to discriminate between  
40 exposure to either low or high quantities of the drug. Over two-thirds (68%) of samples tested  
41 positive for fentanyl at concentrations between LOQ and 8600 pg/mg. The mean value was 382  
42 pg/mg and the median was 95 pg/mg. The metabolites norfentanyl and 4-ANPP were also  
43 quantified and were found between LOQ and 320 pg/mg and between LOQ and 1400 pg/mg,  
44 respectively. The concentration ratios norfentanyl/fentanyl, 4-ANPP/fentanyl, and norfentanyl/4-  
45 ANPP were also tested as potential markers of active use and to discriminate the intake of fentanyl  
46 from other analogs. The common occurrence of samples positive for multiple drugs may suggest  
47 that use is equally prevalent among consumers, which is not the case, as correlations based on  
48 quantitative results demonstrated. We believe this set of experimental observations provides a  
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3 useful starting point for a wide discussion aimed to better understanding positive hair testing for  
4 fentanyl and its analogs in hair samples.  
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8 **Keywords:** hair; interpretation; synthetic opioids; fentalogs; fentanyl  
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For Review Only

## Introduction

Research examining the detection of new psychoactive substances (NPS) in hair has increased in recent years (1–9). Most of the initial barriers to testing, mainly related to the commercial unavailability of reference standards, have now been overcome. Although most NPS fall into the classes of synthetic cannabinoids and designer cathinones, novel synthetic opioids (NSO) have appeared with increasing frequency in the illicit drug supply, leading to a progressive increase in their consumption, which in turn often leads to severe adverse outcomes, including overdose morbidity and mortality (10–14). In fact, in many areas of the United States (US), illicit drugs such as heroin are now commonly replaced or cut with new designer opioids, so many drug users are unintentionally or unknowingly using these new compounds (15–18). To study this phenomenon, toxicologists have developed methods for detecting NSO in hair (19, 20). The analytical methods currently available include a well-known pharmaceutically used substance, fentanyl (21), and many clandestinely-synthesized derivatives. This group of compounds is sometimes referred to as “fentalogs”. Compounds such as sufentanil, alfentanil, remifentanil, and carfentanil (normally approved for human or veterinary use) (21) can now be detected in hair samples, together with other new synthetic opioids such as AH-7921 (22, 23), MT-45 (24), and U-47700 (25–27). The latter are non-fentalogs with different chemical structures which have been involved in intoxication cases and mortality in several countries. Fentanyl and its analogs are typically available in powder form, which can be used directly or mixed with other substances and then smoked or taken via intranasal (28) or intravenous route (29). They can also be pressed into tablets, often as counterfeit forms of other opioid pharmaceuticals (for example, hydrocodone pills (30)), or other drug classes (alprazolam tablets (31)).

One of the challenges in the development of validated methods for the analysis and identification of fentanyl and its analogs in such a rapidly changing and dynamic market is that analytical reference materials may often not be commercially available. Furthermore, interpretation of results is still debated among toxicologists, as is quite common in hair analysis (32). The main matters currently questioned (in order to provide a definitive interpretation of positive vs. negative results) include: 1) the establishment of a reasonable cut-off value for each drug (e.g., in order to discriminate between occasional and frequent use or exposure), and 2) the identification of proper metabolites allowing us to discriminate direct exposure from external contamination. In the present study, preliminary results obtained from a large dataset of real hair samples positive for fentanyl or its analogs are discussed. The findings of this study are meant to provide a useful starting point for a wider

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3 discussion aimed at developing a better understanding of positive hair testing for fentanyl and its  
4 analogs.  
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## 10 **Experimental**

### 11 *Reagents and Standards*

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14 Reagents and standards for furanyl fentanyl and 4-ANPP were produced by Chiron (Trondheim,  
15 Norway); reagents and standards for acetyl fentanyl, remifentanyl and carfentanyl were produced by  
16 Toronto Research Chemicals (North York, Canada); and reagents and standards for tramadol,  
17 oxycodone, hydrocodone and norfentanyl were purchased from Sigma-Aldrich (Milan, Italy).  
18 Reagents and standards for alfentanil, U-47,700, fentanyl, sufentanil and the deuterated internal  
19 standards (norfentanyl-D5, fentanyl-D5 and oxycodone-D6) were produced by Cerilliant (Round  
20 Rock, Texas, US). Ultra-pure water was obtained using a Milli-Q® UF-Plus apparatus (Millipore,  
21 Bedford, MA, USA). All stock standard solutions were prepared in methanol at 1 mg/mL and stored  
22 at -20°C until used. Working solutions were prepared at the final concentration of 1000 ng/mL by  
23 dilution with methanol.  
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### 34 *Sample preparation*

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36 Briefly, about 25 mg of hair was twice-washed with dichloromethane and then methanol (2 mL,  
37 vortex mixed for 3 min). After complete removal of solvent washes, the hair was dried at room  
38 temperature by a gentle nitrogen flow and subsequently grinded with a ball mill (Precellys 24,  
39 Bertin Instruments, Montigny-le-Bretonneux, France). Hair samples were fortified with 2.5 µL of  
40 an internal standards mixture yielding a final concentration of 0.01 ng/mg. After the addition of 1  
41 mL of methanol, the samples were incubated at 55°C for 15 h without stirring. Lastly, the organic  
42 phase was collected and an aliquot of 2 µL was directly injected into the ultra-high performance  
43 liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) system. Whenever the real  
44 sample concentrations were found to exceed the highest calibration point, the final extracts were  
45 diluted with methanol and re-injected into the system.  
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### 56 *Apparatus*

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59 All analyses were performed using a Shimadzu LC-30A Series system (Shimadzu, Duisburg,  
60 Germany), interfaced to an API 5500 triple quadrupole mass spectrometer (Sciex, Darmstadt,

Germany) equipped with an electrospray Turbo Ion source operating in positive-ion mode. A CORTECS UPLC C18 plus column 100 mm × 2.1 mm, 1.6 μm (Waters Corporation, Italy), protected by a C18 waters VanGuard pre-column, was used for target analyte separation. The column oven was maintained at 45°C and the elution solvents were water/formic acid 5 mM (solvent A) and acetonitrile plus formic acid 5 mM (solvent B). After an initial isocratic elution at 95% A for 0.5 min, the mobile phase composition was varied by a linear gradient (A:B; v/v) from 95:5 to 50:50 in 3.5 min; then isocratic elution at 50% B was maintained for 0.5 min. The flow rate was 0.5 mL/min and the total run time was 6 min including re-equilibration at the initial conditions before each injection. MS/MS detection was executed in the selected reaction monitoring (SRM) mode. In order to establish appropriate SRM conditions, each analyte was individually infused into the electrospray ionization (ESI) capillary while the declustering potential (DP) was adjusted to maximize the intensity of the protonated molecular species [M+H]<sup>+</sup>. The collision energy (CE) was set so as to preserve approximately 10% of precursor ion and the cell exit potentials (CXP) were also optimized. The SRM transitions were monitored during a time window of ±17.5 s around the expected retention time, and the cycle time of the SRM program was 0.100 s. Optimal signals were obtained using a source block temperature of 600°C and an ion-spray voltage of 1900 V. Gas pressures were set as follows: curtain gas 35 psi, ion source gas (1) 45 psi and ion source gas (2) 50 psi.

### *Methods*

A simple, fast, specific and sensitive UHPLC-MS/MS method previously developed and validated (19) was applied to detect 13 synthetic opioids (including fentanyl analogs) and metabolites in hair. LOD values were in the range 0.1-0.3 pg/mg for all analytes, with the exception of oxycodone which was 1.5 pg/mg. Samples were collected in the [US](#) between November 2016 and August 2018 from subjects who had reported heroin use in the last year or [were selected among hair samples which had already tested positive for common opiates](#). A total of 293 samples was analyzed. All samples were analyzed in their entire length (range 1-20 cm, mean value 4.0 cm).

### *Statistical analysis*

[Statistical analysis was performed using MATLAB® version 2018b \(MathWorks Inc., Natick, MA, USA\). The data were initially examined with boxplot graphs, before and after logarithmic \(base 10\) transformation. The normality of log<sub>10</sub>-transformed data distributions was checked by the](#)

MATLAB® function “normalitytest”, developed by Öner and Kocakoç, which allowed the simultaneous completion of ten normality tests, including four different versions of Kolmogorov-Smirnov test (i.e., limiting form, Stephens method, Marsaglia method, and Lilliefors test) (33).

The correlation coefficients (r) were calculated on the log<sub>10</sub> transformed and autoscaled data using the MATLAB® built-in “corrcoef” function, which uses the Pearson correlation formula consisting in the ratio of the covariance between two variables over the product of their variance. The obtained coefficients were compared with the ones computed using the Spearman correlation formula applied on the not transformed data.

## Results

### *Prevalence of compounds*

The range of concentrations, including means and medians, were calculated for each analyte. Furthermore, the concentration ratios norfentanyl/fentanyl, 4-ANPP/fentanyl and norfentanyl/4-ANPP were also evaluated as potential discrimination parameters.

Among the 293 samples, two thirds (67.6%; n=198) tested positive to at least one “fentalog” (Table 1). Remarkably, all 198 of these samples tested positive for fentanyl, either alone or together with other molecules. The most common parent compounds detected were fentanyl, acetyl fentanyl and furanyl fentanyl. Concentrations of fentanyl in positive samples ranged between LOQ and 8600 pg/mg. The mean value was  $382 \pm 811$  pg/mg and the median was 95 pg/mg. Over a third (36.8%) of samples (108 samples; 54.5% of those testing positive for fentanyl) tested positive for acetyl fentanyl, with concentrations ranging between LOQ and 3200 pg/mg. The mean value was  $72 \pm 320$  pg/mg and the median was 7 pg/mg. Similarly, 29.7% of samples (87 samples; 43.9% of those testing positive for fentanyl) tested positive for furanyl fentanyl, with concentrations ranging between LOQ and 590 pg/mg. The mean value was  $31 \pm 76$  pg/mg and the median was 6 pg/mg. With regard to metabolites and precursors, norfentanyl was detected in 154 samples (52.6% of all samples and 77.8% of samples positive for fentanyl), with concentrations ranging between LOQ and 320 pg/mg. The mean value was  $38 \pm 55$  pg/mg and the median was 15 pg/mg. Only 21 samples (7.0% of all samples and 10.6% of those testing positive for fentanyl) resulted positive for fentanyl and negative for norfentanyl, all presenting fentanyl concentrations <13 pg/mg. The molecule 4-ANPP was detected in 146 samples (49.8% of all samples and 73.7% of samples



positive for fentanyl), with concentrations ranging between LOQ and 1400 pg/mg. The mean value was  $40 \pm 126$  pg/mg and the median was 8 pg/mg. Box-plots for the most detected compounds are presented in Figure 1. Since the quantitative distribution of the detected compounds was not normal (as evident from Figure 1), a  $\log_{10}$ -transformation of the data was executed prior of calculating the correlation coefficients. All the transformed variables resulted normally distributed, with p-values above the "0.01" significance threshold of for all the Kolmogorov-Smirnov tests (33). Only the Lilliefors test yielded p-values below the threshold ( $p < 0.01$ ) for acetyl fentanyl and hydrocodone. The correlation coefficients from the application of the Pearson formula on the  $\log_{10}$ -transformed and autoscaled data and from the Spearman algorithm on the autoscaled non-transformed data were compared. Since no substantial differences between the two computations were observed, only the results from the Pearson formula are reported (the same applies for the calculations in the following section). In particular, the correlation matrix showing the correlation coefficients between the sets of  $\log_{10}$  transformed and autoscaled variables, namely the concentrations trends for each pair of the screened compounds, is shown in Figure 2. The highest coefficient ( $r = 0.85$ ) was observed for fentanyl and its metabolite norfentanyl.

The most common prescription opioids, including oxycodone, hydrocodone and tramadol, were also monitored. In particular, oxycodone was detected in 168 samples (57.3% of cases), with concentrations ranging between LOQ and 25700 pg/mg. The mean value was  $1003 \pm 2308$  pg/mg and the median was 94 pg/mg.

Three concentration ratios were evaluated. First, the ratio between norfentanyl and fentanyl was in the range 0.01-0.38, with a mean value of 0.10 and a median of 0.08. Then, the ratio between 4-ANPP and fentanyl was in the range of 0.01-6.21, with a mean value of 0.23 and a median of 0.05. Finally, the ratio between norfentanyl and 4-ANPP was in the range of 0.01-25.0, with a mean value of 3.9 and a median of 1.81.

#### *Co-exposure to fentanyl and more common drugs*

A subset of 53 samples positive for fentanyl were also tested for other common drugs of abuse (34, 35). The direct metabolite of heroin, namely 6-monoacetylmorphine, was detected in 49 samples (92.4% of cases), benzoylecgonine, the main metabolite of cocaine, was detected in 45 samples (84.9% of cases), methamphetamine and its metabolite amphetamine were detected in 20 samples (37.7% of cases). With respect to the prescription opioids included in our panel (19), oxycodone or hydrocodone was detected in 35 samples (66.0% of positive cases to fentanyl). The results are

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3 summarized in Table 1. A correlation matrix showing the Pearson correlation coefficients between  
4 the screened compounds (after log<sub>10</sub>-transformation and autoscaling), including the common drugs  
5 of abuse, is shown in Figure 3. In general, relatively low correlation coefficients were calculated  
6 between fentanyl and the other common drugs (ranging from -0.1 to 0.36), with the only exception  
7 of the pair fentanyl/6-MAM yielding the highest coefficient (r = 0.55).

## 15 Discussion

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17 Fentanyl was detected in over two thirds of the tested samples in this analysis. Whenever a sample  
18 was positive for any NSO compound included in the panel of our targeted method, fentanyl was  
19 also always present, suggesting that fentanyl is the most prevalent molecule while the less common  
20 analogs are not consumed in isolation. This is congruent with information obtained from drug  
21 seizures in the US (36). Unlike synthetic cathinones and synthetic cannabinoids, for which the  
22 incessant introduction of newly synthesized drugs has turned analytical methods upgrading into a  
23 “cat and mouse game” (37), for NSO, it seems that laboratories can limit the screening to a few  
24 (prevalent) molecules. Indeed, fentanyl testing is apparently covering the large majority of opioid  
25 misuse, likely without the need to screen for uncommon NSO if mere detection of any exposure  
26 (positive vs. negative) is the ultimate goal of hair analysis.

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28 The fentanyl analogs most frequently detected were acetyl fentanyl and furanyl fentanyl, both  
29 showing a lower mean concentration with respect to fentanyl. While the former can often be present  
30 as an impurity in fentanyl preparations, it is likely that the latter was used more directly, possibly as  
31 an adulterant. These two illicit compounds were the compounds most commonly observed at the  
32 time of our sample collection in 2016-2018 (36), while other designer fentanyl analogs were only  
33 rarely detected during this time period. However, the drug landscape continues to evolve rapidly,  
34 making it necessary to periodically update analytical methods in order to periodically add additional  
35 target NSO (e.g., 3-methylfentanyl, butyrylfentanyl) to keep the pace with the introduction of new  
36 drugs into the black market.” Remarkably, carfentanil, representing the NSO of the highest concern  
37 because of its pharmacological potency (estimated at being some thousands of times more potent  
38 than morphine), was detected in only four samples, at very low concentrations. The possible reasons  
39 for the rare occurrence of samples testing positive to carfentanil are: 1) the low prevalence at the  
40 time of the sample collection, 2) poor incorporation or low stability of carfentanil in the keratin  
41 matrix, and/or 3) insufficient sensitivity of the analytical method in relation to the low effective  
42 dosage. On the other hand, fentanyl itself is possibly potent enough to exclude the need of

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3 introducing further potentially lethal substances into the illegal market. Indeed, more investigation  
4 is still needed before a final interpretation is given for the low occurrence of carfentanil in hair  
5 testing.  
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9 Fentanyl and the other NSO analogs were quantified in a wide range of concentrations. Several  
10 factors can account for the large variability observed in the data, as it is typical for hair analysis and  
11 more in general for the incorporation of drugs into the keratin matrix collected from different  
12 subjects. (9). The most important influencing factor is obviously the ingested doses, together with  
13 the frequency of drug use. Other inter-individual parameters affecting the measured concentration  
14 in hair include the melanine content (particularly for basic and hydrophobic substances), the  
15 individual's metabolism, and other behavioral and environmental factors (9). Median and box-plot  
16 calculations were used to draw a preliminary direction for a possible cut-off to discriminate between  
17 exposures to either low or high quantities of drugs. The median value for fentanyl was 95 pg/mg, a  
18 value not far from the 200 pg/mg cut-off universally used for opiates (38–40). The main metabolites  
19 could also be detected in the majority of hair samples supporting the possibility to ascertain active  
20 use. In particular, norfentanyl could be detected in most samples positive for fentanyl. Only in the  
21 samples with particularly low fentanyl concentrations, namely <13 pg/mg, norfentanyl could not be  
22 detected. Thus, this compound proved to be present in increasing concentrations when the fentanyl  
23 concentration was high. The strong correlation (coefficient of 0.85) between fentanyl and its  
24 metabolite norfentanyl, also in terms of concentration trends, is clearly visible. Another promising  
25 marker for the fentalog class is 4-ANPP. This compound is frequently detected in real samples,  
26 about as often as norfentanyl. However, its presence can be attributed not only to metabolism, but  
27 also to chemical synthesis, since 4-ANPP is a common precursor of fentanyl and several of its  
28 analogs. Consequently, it is conceivable to use 4-ANPP as a class-marker when screening for  
29 fentalog. In this scenario, the simple detection of 4-ANPP in a real sample could provide evidence  
30 of fentalog intake, whether exposure is known or unknown.  
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48 Three ratios were also evaluated. We believe the ratio norfentayl/fentanyl represents a promising  
49 marker to ascertain active use and discriminate the intake of fentanyl from other analogs. The  
50 higher the ratio, the more likely the individual has been exposed to fentanyl. The ratio for 4-  
51 ANPP/fentanyl is difficult to interpret, however, since 4-ANPP may originate from the synthesis  
52 and/or the metabolism of fentanyl. The ratio for norfentanyl/4-ANPP could lead to different  
53 interpretations. A low ratio corresponding to high 4-ANPP concentration might indicate external  
54 contamination of fentanyl, producing higher quantities of the precursor than the metabolite. On the  
55 other hand, a high level of 4-ANPP and a low level of norfentanyl could also be produced by the  
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3 intake of fentanyl analogs, corresponding to a different metabolism but a similar chemical  
4 synthesis.  
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7 On the positive samples, the observed median value for oxycodone was close to the median value of  
8 fentanyl, despite the fact that fentanyl-based pharmaceutical preparations usually contain amounts  
9 of active drug 1/10-1/100 lower than those contained in oxycodone preparations. Surely the  
10 structural differences between the two molecules may result in different physical and chemical  
11 properties, which in turn influence the incorporation into the keratin matrix. However, the fact that  
12 the two populations have similar distributions and close median values (despite the pharmaceutical  
13 preparations available on the market having very different amounts of active principle), this may  
14 further support the hypothesis that the investigated population had misused fentanyl, namely above  
15 the therapeutic dosages.  
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24 Another source of information is provided by the co-presence of traditional drugs of abuse and  
25 prescription opioids. The large majority of the tested subjects were positive for fentanyl and heroin  
26 and/or cocaine simultaneously. It is not possible to deduce whether fentanyl was used separately  
27 from heroin or cocaine, or whether the heroin or cocaine was cut with fentanyl. However, from a  
28 quantitative perspective, relatively low correlation coefficients may suggest that subjects who were  
29 more exposed to fentanyl were less exposed to heroin or cocaine, and vice versa, probably due to  
30 different sources of the substances. More in general, our findings confirm that many of the subjects  
31 who use illegal drugs are (perhaps repeatedly) exposed to several substances, knowingly or  
32 unknowingly. In particular, the subset of considered samples indicated that the intake of cocaine  
33 was only scarcely correlated with that of fentanyl. Within the class of prescription drugs, a  
34 significant percentage of subjects tested positive for both fentanyl and oxycodone and/or  
35 hydrocodone, even though the correlation coefficients are relatively low, indicating the preferential  
36 consumption may be of a single substance, at least during the period of time corresponding to the  
37 hair length. This finding may be of interest because it appears to be congruent with one explanation  
38 of the third wave of opioid deaths (11). According to this theory, the recent increase of fentanyl and  
39 heroin overdoses is linked to at-risk users who have been driven to fentanyl and heroin in response  
40 to lessened availability of milder prescription opioids such as tramadol, oxycodone, and  
41 hydrocodone, thus exposing themselves (perhaps unknowingly) to the even more dangerous family  
42 of fentalogs.  
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## Conclusions

In the gradual interpretation process of hair testing results for a new class of abused substances such as NSO, much more experimental data are needed before the routes and mechanisms of hair incorporation are clarified, the metabolic pathways are fully understood, and potential discrimination factors are identified [9]. However, we believe that the present study provides a useful yet preliminary insight into these issues to start a wider discussion aimed to better understand the positive hair results observed for fentanyl and analogs.

As long as fentanyl is a commercially available medicinal drug, there is a fine line between detectability of therapeutic use vs. illicit use or abuse. However, our preliminary data suggest an approximate cut-off value around 100 pg/mg to realistically discriminate between therapeutic intake of fentanyl and its abuse.

When the possible occurrence of external contamination is claimed to justify a positive test result we believe the detection of the metabolite norfentanyl will sustain the hypothesis of active intake. On the other hand, 4-ANPP may represent a useful marker to identify the intake of either fentanyl or other fentanyl analogs, since it is found as both a fentanyl metabolite and as an impurity produced in the synthesis of fentanyl analogs.

Hair analysis shows that the parent drug – fentanyl - is still the largely predominant abused NSO drug in the tested population. Fentanyl abuse is frequently associated with the use of heroin and other synthetic NSO (acetyl fentanyl, furanyl fentanyl), more rarely associated with the consumption of milder therapeutic NSO (tramadol, oxycodone) and seldom associated with use of cocaine. From a general perspective, hair analysis proved once again to represent a fundamental approach to understand new phenomena of drug abuse, drug diffusion among selected populations on geographical or sociological basis, and drug use patterns, prevalence, and potential unintentional exposure as substitution/cutting drug, or impurity.

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## Conflict of interest

The authors declare no conflict of interest.

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**Table 1** Summary of results obtained from 293 real hair samples

Target analyte	Number of positive samples	Range of concentrations (pg/mg)	Mean	Median
Fentanyl	198	LOQ-8600	382	95
Acetyl fentanyl	108	LOQ-3200	72	7
Furanyl fentanyl	87	LOQ-590	31	6
Norfentanyl	154	LOQ-320	38	15
4-ANPP	146	LOQ-1400	40	8
U-47700	22	LOQ-420	44	5
Carfentanyl	4	LOQ-1.5	1.1	1.2
<b>Prescription opioids</b>				
Hydrocodone	211	LOQ-12600	269	24
Tramadol	201	LOQ-34700	606	30
Oxycodone	168	LOQ-25700	551	94
<b>Other drugs of abuse (results in ng/mg, obtained from 53 samples positive to fentanyl)</b>				
Heroin metabolites	49	0.06-6.94	0.64	0.18
Cocaine metabolites	45	0.05-7.95	1.65	1.52
Amphetamines	20	0.05-6.01	1.78	1.42

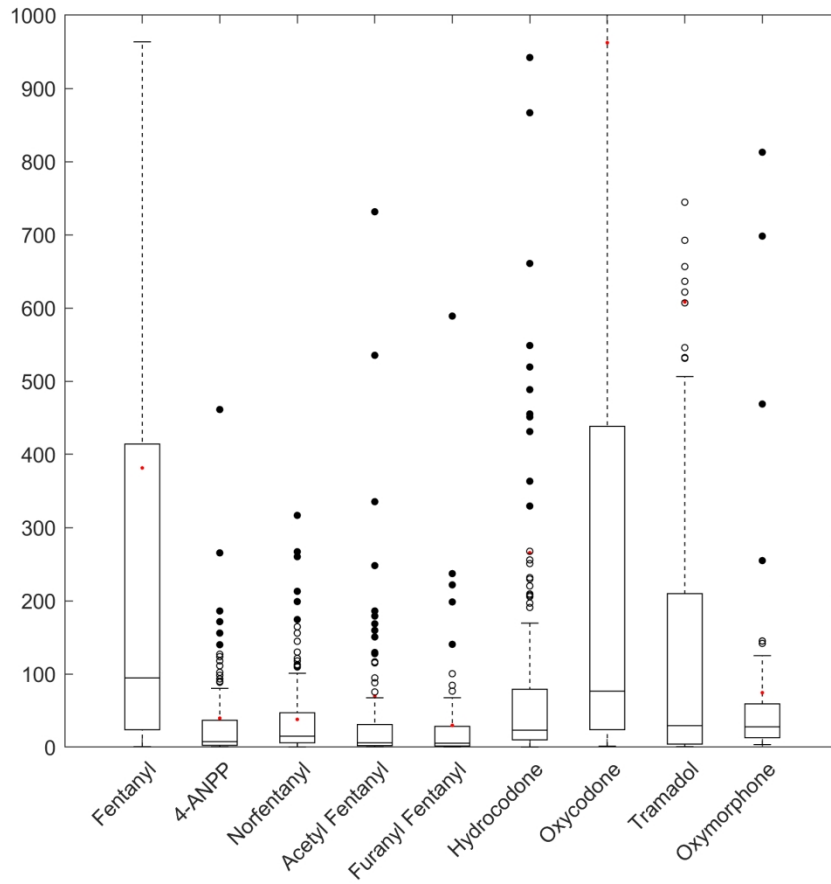
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3 **Figure 1** Box-plots for the most detected compounds  
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5 **Figure 2** Correlation matrix for 198 samples positive to fentanyl  
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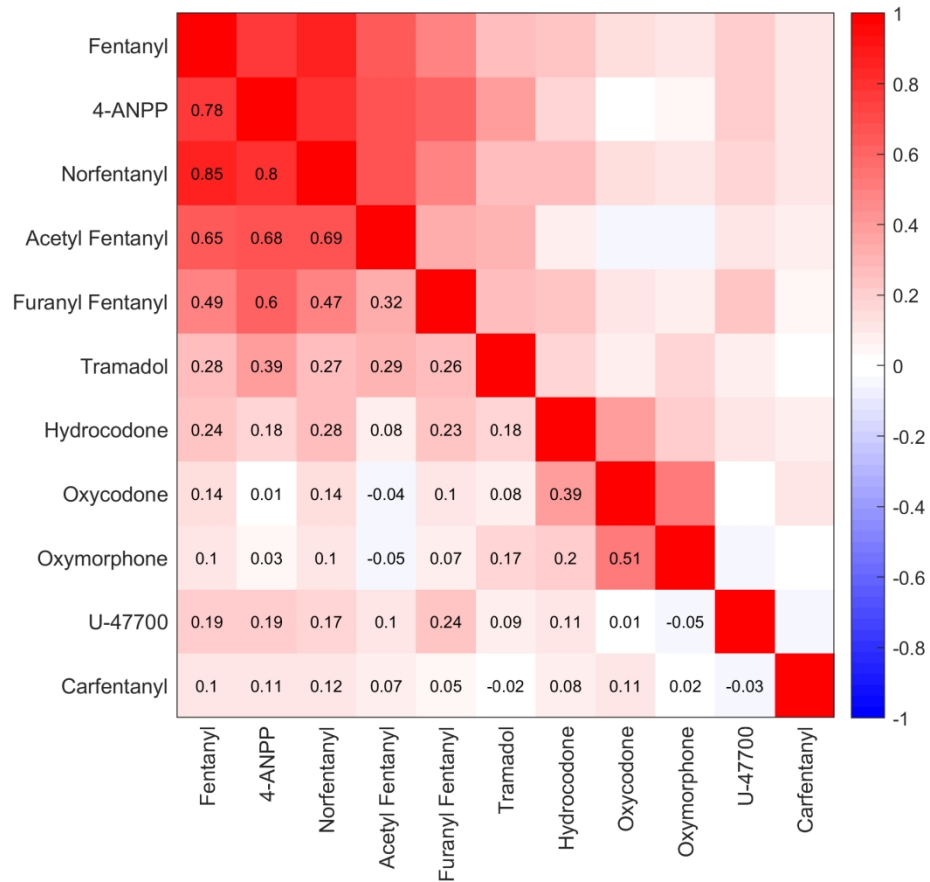
7 **Figure 3** Correlation matrix for 53 samples positive to fentanyl tested also for common drugs of  
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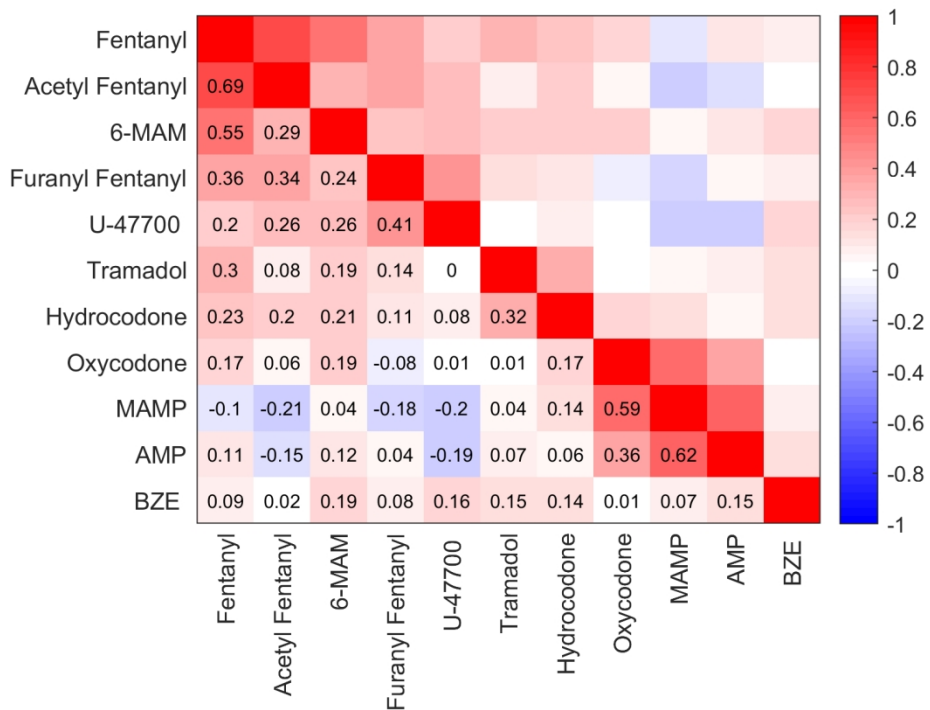
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