**Interpretation of NPS results in real hair samples**

Elsevier Editorial System(tm) for
Toxicologie Analytique et Clinique
Manuscript Draft

Manuscript Number: TOXAC-D-16-00055R1

Title: Interpretation of NPS results in real hair samples

Article Type: Revue générale / Review Article

Section/Category: Toxicologie analytique / Analytical Toxicology

Keywords: Hair; new psychoactive substances; synthetic cannabinoids; synthetic cathinones; interpretation

Corresponding Author: Dr. Alberto Salomone, Ph.D.

Corresponding Author's Institution: Centro Regionale Antidoping e di Tossicologia

First Author: Alberto Salomone, Ph.D.

Order of Authors: Alberto Salomone, Ph.D.; Marco Vincenti; Enrico Gerace

Abstract: Today, the use of hair analysis for forensic issues concerning drugs collectively indicated as NPS is still controversial. In particular, little is known about the incorporation into the keratin matrix after intake and the correlation between their dosage, passive exposure, use frequency, and hair concentrations. In the present review, we considered the main issues which still deserve substantial research and discussion within the scientific community, before a definitive interpretation of either a positive or negative results can be safely given to the local authorities. Specifically, the following scenarios were considered: i) passive exposure vs. active consumption, ii) mindful vs. unaware intake, and iii) sporadic vs. chronic use. Differently from the traditional drugs of abuse, whose chemical and toxicological properties have been largely elucidated, in the context of NPS the range of chemical structures is so various that it is difficult to speculate about general criteria. Under these circumstances, any analytical outcome from NPS hair analysis should be cautiously interpreted by experienced forensic toxicologist.

# Interpretation of NPS results in real hair samples

Alberto Salomone[1,*] , Marco Vincenti[1,2] , Enrico Gerace[1]

[1]*Centro Regionale Antidoping e di Tossicologia "A. Bertinaria", Regione Gonzole 10/1, 10043 Orbassano, Turin, Italy*

[2]*Dipartimento di Chimica, Università di Torino, Via Giuria 5, 10125 Turin, Italy*

[*]**Corresponding author**: Alberto Salomone
Centro Regionale Antidoping "A. Bertinaria", Regione Gonzole 10/1 - 10043 Orbassano, Torino, Italy
Email: alberto.salomone@antidoping.piemonte.it
Tel.: +39.011 90224232; FAX.: +39.011 90224242; Mobile: +39 3489330145

**ABSTRACT**

Today, the use of hair analysis for forensic issues concerning drugs collectively indicated as NPS is still controversial. In particular, little is known about the incorporation into the keratin matrix after intake and the correlation between their dosage, passive exposure, use frequency, and hair concentrations. In the present review, we considered the main issues which still deserve substantial research and discussion within the scientific community, before a definitive interpretation of either a positive or negative results can be safely given to the local authorities. Specifically, the following scenarios were considered: i) passive exposure vs. active consumption, ii) mindful vs. unaware intake, and iii) sporadic vs. chronic use. Differently from the traditional drugs of abuse, whose chemical and toxicological properties have been largely elucidated, in the context of NPS the range of chemical structures is so various that it is difficult to speculate about general criteria. Under these circumstances, any analytical outcome from NPS hair analysis should be cautiously interpreted by experienced forensic toxicologist.

**Keywords:** Hair; new psychoactive substances; synthetic cannabinoids; synthetic cathinones; interpretation

## INTRODUCTION

Around a decade ago, the first reports were published that described the existence of an unprecedented class of "new psychoactive substances" (NPS) distributed under the misleading name of "legal highs", "designer drugs", "herbal highs", "bath salts" or "research chemicals" (1,2). Immediately after, forensic and clinical laboratories worldwide had to face the analytical challenge of the identification and quantification of these new drugs in various biological matrices. The commercial unavailability of reference standards for the parent drugs and their metabolites, the lack of updated and comprehensive immunoassays for their detection, and the extensive, yet insufficient, investigation of their metabolic transformation after intake, represented just the main problems toward the identification and quantification of NPS (3,4).

Nowadays, most of the aforementioned challenges have been overcome, at least partially. Several laboratories are offering screening and confirmation analysis for NPS in the context of workplace drug testing, driving re-licensing, roadside control and withdrawal programs. The identification of a certain NPS in urine and blood proves the recent exposure to this substance, enabling the authorities to prosecute and/or sanction the sample donor according to the local legislation. On the contrary, the use of hair analysis (in general, any keratin matrix) for forensic issues concerning NPS is still argued. The simple detection of NPS in hair has been extensively described in a recent book by one of the authors (3). However, the results interpretation presents several controversial issues, as is quite common in hair analysis (3,5,6). In the present review, we considered the main matters currently under debate, which still deserve substantial research and discussion before a definitive interpretation of either positive or negative results can be safely given. Specifically, the following alternative circumstances were considered: i) passive exposure vs. active consumption, ii) mindful vs. unaware intake, and iii) sporadic vs. chronic use.

For consistency, the terms "synthetic cathinones" and "synthetic cannabinoids" were exclusively used. Drugs frequently classified under different categories or with different chemical structure were regrouped in an attempt to obtain comprehensive classification of similar drug subsets. Other groups of new designer drugs (e.g. opioids and designer benzodiazepines) were not included because their detection in hair has been reported only sporadically until today. Therefore, any comment about the interpretation would appear hasty.

## PASSIVE EXPOSURE VS. ACTIVE INTAKE

The most common administration routes for synthetic cathinones are insufflation (snorting) and oral ingestion of capsules or tablets, or powder wrapped in cigarette paper and swallowed (so-called ‘‘bombing’’). Quite often, the substance is dissolved or diluted with water/juice drink, to give an intoxicating beverage. Rectal insertion, intravenous, subcutaneous, and intramuscular injections are less frequently reported (7,8). On the other hand, synthetic cannabinoids are predictably smoked. For both classes of NPS (synthetic cathinones and synthetic cannabinoids), external contamination is possible, especially when the drug is handled in relatively big amounts, as it may occur to drug dealers and police officers, so that contamination from hand contact or residues on furniture surfaces – like in homes formerly used as a clandestine drug laboratory- is likely and needs to be taken in consideration (9–15). As for other biological matrices, the identification of metabolites is recommended also in hair analysis (16,17), as the only way for the exclusion of external contamination in most cases.

Synthetic cathinones

In 2012, Shah et al. developed a LC-MS/MS method for the quantitative analysis of mephedrone and two of its metabolites, namely 4-methylephedrine and 4-methylnorephedrine, in hair (18). The authors screened the hair of 154 healthy volunteers for mephedrone, but only five samples tested positive, four of which at very low level. The metabolites were not detected (LOD: 5 pg/mg) in any of the analyzed samples while mephedrone could be successfully quantified in only one sample at a concentration of 21.1 pg/mg. In no case, it had been possible to confirm the positive result with the donor’s admission of mephedrone use. The authors also noted that contamination from environmental exposure was not likely because mephedrone is not smoked.

In a recent paper (19), the concurrent detection of mephedrone, a mephedrone isomer (namely 3-methylmethcathinone) and two metabolites (3-methylephedrine and 3-methylnorephedrine) in pubic hair samples was obtained by means of liquid chromatography–high resolution/high accuracy Orbitrap mass spectrometry. In the presented case, a man was charged with dealing of NPS-containing materials. The powders and tablets seized by police contained 4-methylethcathinone (4-MEC), 3-methylmethcathinone (3-MMC), α-methylaminovalerophenone (pentedrone), 6-(2-aminopropyl) benzofuran(6-APB), 1-(benzofuran-5-yl)-N-methylpropan-2-amine (5-MAPB). The alleged drug dealer claimed that he was also a consumer of such drugs, and he intended to demonstrate his personal use for legal purposes. The pubic hair sample of the drug dealer turned out to be negative for 4-MEC, pentedrone, 6-APB, and 5-MAPB following LC-HRMS analysis. Conversely, 3-MMC was detected in pubic hair and quantified at a concentration of 25.8 ng/mg. The authors also sought to detect any metabolite of 3-MMC in the pubic hair sample of the drug

dealer and actually identified 3-methylnorephedrine, 3-methylpseudonorephedrine, 3-methylephedrine and 3-methylpseudoephedrine. Their estimated concentrations were about one third (3-methylephedrines) and one thirtieth (3-methylnorephedrines) of 3-MMC concentration. This is consistent with the results usually observed in toxicological hair analysis, due to the lower incorporation of polar metabolites in the hair matrix with respect to the parent compounds. The metabolites' detection enabled to prove personal use of 3-MMC by the drug dealer, even though pubic hair contamination from urine cannot be completely excluded.

Synthetic cannabinoids

As for $\Delta^9$-tetrahydrocannabinol, the mere detection of any parent synthetic cannabinoid in hair samples does not exclude the possibility of external contamination, nor does it provide conclusive evidence of active drug consumption. As a matter of fact, only the detection of their metabolites and possibly the evaluation of concentration ratio between parent drugs and metabolites can sustain the active use of synthetic cannabinoids and in most cases exclude external contamination from side stream smoke or material handling.

The first studies to investigate the presence of NPS metabolites in hair were presented by Kim et al (20,21). In their first study (20), the authors validated an analytical method for simultaneous detection of JWH-018 and JWH-073, and their most abundant mono-hydroxylated and carboxylated metabolites. The method was applied to 18 hair samples from individuals suspected of using synthetic cannabinoids. Among the positive results, only the N-(5-hydroxypentyl) metabolite of JWH-018 (JWH-018 N-5-OH) was found, suggesting its prevalence in hair. Its concentrations were found to vary over a wide range, and the same was recorded for the ratio between parent drug and metabolite. Even some hair containing relatively high concentrations of JWH-018 (above 50 pg/mg), did not show the presence of JWH-018 N-5-OH. The highest concentration recorded for JWH-018 N-5-OH was 85 pg/mg, corresponding to a JWH-018 concentration of 151 pg/mg. Overall, in samples positive to both JWH-018 and JWH-018 N-5-OH, the parent drug-to-metabolite ratio was highly variable, ranging from 1.1 to 62.8. Noteworthy, JWH-018 N-5-OH is also the main product of AM-2201 metabolism (22), opening up the chance that particularly high concentrations of JWH-018 N-5-OH in hair may be generated by the concurrent ingestion of several NPS. In conclusion, the results of JWH-018 N-5-OH levels in hair are at the moment inconclusive and a comprehensive metabolite screening of the most popular synthetic cannbinoids, including AM-2201 appears to be necessary. In the second study of the same group (21), the method previously developed was extended to AM-2201, JWH-122, MAM-2201 and their mono-hydroxylated metabolites in hair. The method was also applied to investigate the distribution in authentic human

hair samples taken from real forensic cases and the relative incorporation rate of AM-2201 and its metabolites in pigmented and non-pigmented rat hair. In real samples, JWH-018, JWH-018 N-5-OH, JWH-018 N-COOH, JWH-073, JWH-073 N-COOH, AM-2201, AM-2201 N-4-OH, AM-2201 N-6-OH indole, JWH-122, JWH-122 N-5-OH and MAM-2201 were detected, either simultaneously or individually. The concentration range of the parent drugs (e.g. AM-2201) was found to be much larger than that of the corresponding metabolites (e.g. JWH-018 N-5-OH). The parent synthetic cannabinoids and their mono-hydroxylated metabolites were identified in the hair samples of all nine cases, confirming that the simultaneous determination of both parent drug and metabolites in hair is helpful for most synthetic cannabinoids in order to exclude the possibility of passive contamination and also provide valuable information about the spectrum of molecules potentially ingested (21).

The issue of possible external contamination has also been raised by several Authors (9,23,24). In the first study (9,23), the extent of external contamination caused by handling drug material containing synthetic cannabinoids under realistic conditions was evaluated in a forensic laboratory. Hair samples from laboratory workers involved in the analysis of 670 herbal mixtures (covering 31 brands and 12 synthetic cannabinoids) within a two-weeks period were tested for synthetic cannabinoids with a validated LC-MS/MS method. In addition, hair samples from laboratory staff not working in direct contact with the drug material and close relatives of the exposed subjects were analyzed to check for cross contamination. All samples of workers in direct contact with the drug material tested positive for at least one synthetic cannabinoid. The measured concentrations ranged from trace level up to a maximum of 170 pg/mg (JWH-210) and roughly reflected the duration of exposure. Unexpectedly, some subjects not having direct contact to the drug material also showed measurable drug concentrations in hair. In one case, the JWH-210 concentration measured in the hair sample of a worker who was involved in the study was less than 0.5 pg/mg, whereas that detected in the hair sample of his girlfriend, who lived in the same household, but had no contact with the drug materials, was up to 11 pg/mg. Overall, the hair drug concentrations determined by mere external contamination were found in the range overlapping that typical for known drugs users, even if the majority of them was below 50 pg/mg. It was concluded that the actual consumption of synthetic cannabinoids can be unquestionably proved only by the detection of their metabolites in hair or, alternatively, the simultaneous positive testing of either urine, blood, or oral fluid.

In another study, the concentrations of synthetic cannabinoids were measured in scalp hair after exposure to side-stream smoke from a cigarette containing JWH-018, JWH-122, and JWH-210

(24). The study showed that synthetic cannabinoids remain linked to the hair shaft long time after exposure to side stream smoke. Since these substances cannot be completely removed by routine washing procedures prior to analysis, and the concentration ratio between wash solutions and hair extracts does not necessarily reflect external contamination, the positive results caused by side stream smoke exposure can lead to erroneous conclusions.

The reliability of hair testing to reveal synthetic cannabinoid abuse was also questioned by Saito et al (25), whose study proved that significant adsorption of synthetic cannabinoids occur in the hair of a non-user after passive exposure. The authors exposed both cosmetically treated and untreated scalp hair by soaking with NNEI and MAM-2201 aqueous solutions. The experiments showed that both compounds were partially adsorbed by both untreated black and dyed hair and were not easily eliminated by means of a regular washing procedure.

A further study (26) was aimed to develop and validate an analytical method for simultaneous detection of XLR-11 and its metabolites in hair. The method was applied to investigate the distribution of XLR-11 and its metabolites in 14 authentic human hair samples: XLR-11, UR-144, UR-144 N-5-OH, UR-144 N-COOH, and XLR-11 N-4-OH were detected with widely variable distributions of their quantitative results.

Recently, the first results from a comprehensive screening of drugs and metabolites on a large group of subjects were presented (27) and later expanded to a larger population (3). Overall, 23 samples were tested positive for synthetic cannabinoids. In 16 cases, low concentrations of the parent drug (below 50 pg/mg) were measured and no metabolites were found. For these cases, sporadic exposure represent the most likely event, even if external contamination could not be excluded. In four cases, samples were tested positive for multiple drugs at relatively high concentrations (above 50 pg/mg), but negative for metabolites. Frequent exposure to synthetic cannabinoids is the most likely explanation, even though no metabolites were found. The remaining records included three samples that exhibited very high concentrations for different synthetic cannabinoids and also some metabolites at very low concentration. The latter cases are fully compliant with frequent exposure to synthetic cannabinoids, proven by (i) high levels, (ii) multiple positive testing, and (iii) presence of metabolites.

It is not unrealistic that certain known NPS metabolites can be formed also by different routes, not involving metabolic transformations. To evaluate several aspects possibly affecting hair analysis results for synthetic cannabinoids, the stability of 5F-PB-22 and AB-CHMINACA was assessed by analyzing smoke condensates and hair samples stored under different conditions (28). For comparison, an authentic hair sample of a patient with known history of heavy synthetic

cannabinoids consumption was analyzed in segments. In all of them, 5F-PB-22 and AB-CHMINACA were detected, together with three metabolites: 5F-PB-22 3-carboxyindole, PB-22 5-OH-pentyl, and AB-CHMINACA valine. Alternative origins of these substances was investigated in smoke condensates and contaminated hair (by 5F-PB-22 and AB-CHMINACA) after thermal stress. In smoke condensates, both 5F-PB-22 3-carboxyindole and AB-CHMINACA valine were detected. This experiment proved that both products can be formed as pyrolytic artifacts during smoking, and indicated side-stream smoke as a possible source of contamination for these alleged metabolites. On the other hand, the thermally treated contaminated hair samples also showed the presence of 5F-PB-22 3-carboxyindole, PB-22 5-OH-pentyl, and AB-CHMINACA valine, along with their respective parent compounds. Again, it was demonstrated that both drugs undergo hydrolysis after deposition onto the hair, preventing clear distinction between true metabolites and artifacts.

## MINDFUL VS. UNAWARE INTAKE

Quite remarkably, the majority of the published studies describing NPS detection in real hair samples, reported the frequent occurrence of poly-abuse (29–33). Actually, no systematic correspondence exists between herbal blend trade name and real content in synthetic cannabinoids, making the consumers rarely aware of the actual composition of the purchased products. This is not surprising, because herbal blends are not standardized products, but rather semi-clandestine preparations obtained from mixtures of herbal leaves of different origin, on which various synthetic cannabinoids are sprayed. According to the cannabinoids availability, the active ingredients may vary from lot to lot, even when the trade name is the same. It was also shown that cannabinoids concentration may vary significantly among different packages of the same brand (34–37) and that some blends may contain two or more active compounds (35,38–40). Variable amounts and combinations of these ingredients are therefore put together in “Spice” products to generate cannabis-like effects. It is not uncommon that further substances with similar or different pharmacological activity are added to herbal mixtures (3,41). This inconstancy of the “legal highs” contents implicate that consumers do not have control the power and the effects of the product they are consuming (38).

Likewise, it has been shown that pills or powders purchased as stimulants drugs often contain more substances than is declared on the label (7,35). Doubts about drug identity, purity, actual dosage, multiplicity, and potential synergistic effects are fundamental unanswered questions that expose NPS users to hazardous consequences (35). Jang et al (42) reported a fatal poisoning involving paramethoxyamphetamine (PMA). Upon hair analysis, PMA was detected at a concentration of

20.1 ng/mg, suggesting consumption of this drug in the two months before death. Ketamine and MDMA were also detected at high concentrations in the same specimen. The authors speculated that the deceased drug user may have ingested PMA expecting to be taking MDMA, as it occurred in many other PMA-related fatal cases.

An innovative approach to gain information on NPS diffusion combined questionnaire administration and hair sampling from nightclub/festival-attending young adults (age 18–25) in New York City (43). Out of participants who accepted to donate an hair sample, 48 also reported habitual use of ecstasy/MDMA/Molly. Half (50.0%) of hair samples actually contained MDMA, 23 contained butylone (47.9%), and 5 methylone (10.4%). Of those who reported no lifetime use of any "bath salts", stimulant NPS, or unknown pills or powders, 41.2% tested positive for butylone, methylone, alpha-PVP, 5/6-APB, or 4-FA, suggesting that many ecstasy-users among nightclub/festival attendees may unintentionally purchase "bath salts" or other NPS in place of MDMA. In another study (44), 23 real samples taken from proven MDMA and ketamine abusers were tested for the presence of 31 NPS, among cathinones and other stimulant, psychedelic and dissociative designer drugs. Some NPS were detected in multiple samples, in particular methoxetamine (3 samples, range of concentration: 7.7-27 pg/mg), mephedrone (2 samples, respectively 50 and 59 pg/mg), while other drugs were identified in a single sample: 4-MEC (330 pg/mg), methylone (<LOQ), α-PVP (1040 pg/mg), 4-FA (55 pg/mg), MDPV (120 pg/mg) and diphenidine (4400 pg/mg), proving past poly-abuse of several NPS.

**SPORADIC VS CHRONIC USE?**

The range of chemical structures collectively indicated as NPS is so various that it is difficult to determine or even speculate about the binding capacity of each single substance to the keratin matrix. On the other hand, the pharmacological potency of NPS tested *in vitro* is generally extremely high, and it is believed that these compounds are extremely active at relatively low doses also *in vivo*. The low dosage at which high-potency NPS are taken reduces the chance to find detectable levels in hair even under the favorable circumstance that the drug has high affinity toward the keratin matrix. Lack of knowledge about both effective dosage of NPS and their keratin binding make any attempt to establish a reasonable cut-off value for each drug questionable. Some laboratories set the limit of detection as the minimum hair concentration to ascertain the use of NPS, but this practice does not settle the matter if cut-off values should be used in hair analysis in order to discriminate between chronic consumption and occasional use (or even single scouting intake). At the moment, only few papers dealt with the expected NPS concentration in real samples,

and even less studies have investigated the relation occurring between detected levels and frequency of use.

One of the first papers that reported the detection of NPS in hair (45) found thirteen samples positive for mephedrone, with relatively high concentrations (range: 0.2-313.2 ng/mg, mean: 26.8 ng/mg). The authors concluded that mephedrone is likely to be extensively incorporated into hair, like other stimulant drugs such as amphetamines or cocaine.

In a case of a 25-year-old man found dead in the apartment of a friend (46), hair analysis revealed past exposure to mephedrone (0.25 ng/mg). This finding was consistent with the statement of the decedent's friend, reporting that he was a consumer of new designer drugs.

Wikström et al (47) described two fatal intoxications with the new designer drug methedrone (4-methoxymethcathinone). In the first case, hair was not sampled during the autopsy. In the second case, short hair segments (three 5-mm and two 10-mm segments) revealed an even distribution of methedrone (segment one: 37 ng/mg, segment two: 33 ng/mg, segment three: 29 ng/mg, segment four: 29 ng/mg, and segment five: 36 ng/mg), suggesting chronic intake of methedrone over the months preceding the intoxication.

A method for the detection of 4-MEC and MDPV was validated and applied to a 30-year-old man who usually consumed cathinones for 6 months administered intravenously (48). Both 4-MEC (30 ng/mg) and MDPV (1 ng/mg) were identified in the hair at high concentrations showing a regular consumption of these drugs.

The detection of the dissociative anesthetic designer drug diphenidine in hair was recently described. In a case of a first time use (49), a hair sample was obtained 49 days after ingestion, and was divided into five 1 cm segments. Diphenidine was identified on the first three proximal segments, probably due to sliding hair in the strand, at concentrations of 123, 79 and 89 pg/mg.

In another case, diphenidine was detected at 4400 pg/mg concentration in the hair sample of a 30-year-old Caucasian man with previous history of drug addiction (50). According to the authors, the high hair concentration indicates that the subject had previously been exposed to diphenidine in several occasions.

An interesting discussion about the incorporation of NPS in hair was proposed by Namera et al (51), in a report dealing with the detection of pyrrolidinophenone-type designer drugs (such as MDPV and α-PVP) in patients suspected to consume illegal drugs. The authors speculated that pyrrolidinophenone-type designer drugs should be expected to have a low incorporation rate into

the keratin matrix because of the beta-carbonyl group in their structure. In contrast, α-PVP was found in several segments from different subjects, with the correspondent highest hair concentrations ranging from 10 to 300 ng/mg. They concluded that the positive binding effect of the pyrrolidine group due to its hydrophobic character may exceed the negative effect of the beta-carbonyl group. Also MDPV was detected in this study as well as in another report from the same authors (52), at even higher concentrations, in which hair samples were collected from a 35-year-old woman found unconscious and pronounced dead at the hospital. Segmental analysis was conducted to confirm chronic drug abuse. MDPV and α-PVP were detected in ten and five consecutive 10-mm segments, respectively, with the highest MDPV concentration found at 22 ng/10-mm hair. Analogous investigations about the rate of incorporation of NPS were proposed by Nieddu et al. (53), who evaluated the different accumulation of some target phenethylamines occurring between pigmented and non-pigmented rat hair. Concentrations above 0.20 ng/mg were detected only in pigmented hair.

Also for synthetic cannabinoids, several studies dealt with real samples from forensic casework but only rarely the analytical determination was completed with information about the frequency of past use. Hutter et al (30) obtained hair samples from a population of forensic psychiatry patients who admitted chronic consumption of several herbal mixtures in the last few months before sampling. The self-stated intake frequency ranged from three times during six weeks up to daily consumption of half a package for seven months. Eight samples tested positive for several synthetic cannabinoids, with concentrations ranging from 0.5 to 78 pg/mg. Other studies presented real hair sample analysis (29,31–33,54,55), yielding a broad range of concentrations encompassing 3-4 orders of magnitude but no accessory information about past use. From these studies, it was hardly possible to draw any conclusion about the possible correlation between the NPS use history and the concentrations measured in hair. Although the cut-off level of 50 pg/mg is internationally accepted for THC in hair, there are no enough data in current literature to draft similar conclusion for synthetic cannabinoids. Further studies should be performed to discriminate between sporadic and chronic use, particularly when the concentrations detected are lower than 50 pg/mg (29).

## CONCLUSIONS

For several decades, the studies concerning drugs of abuse were restricted to a very limited number of substances, whose chemical and toxicological properties were progressively elucidated. In contrast, hundreds of NPS came to prominence in the last decade, making it impossible to determine for each one the chemical, pharmacological, and toxicological properties, together with

their biological distribution and metabolic fate. Unfortunately, even less is known about their incorporation into the keratin matrix after intake and the correlation between their dosage, use frequency, and hair concentrations. Under these circumstances, any possible conclusion that a forensic toxicologist may draw from hair analysis about NPS abuse have high chance to be seriously questioned in court, particularly when the detected hair concentrations are lower than 50-100 pg/mg. For this reason, it is important (i) to interpret hair testing data about NPS with extreme care, (ii) to use published studies whenever possible, but keeping any conclusion strictly connected with the context under examination, (iii) to develop original, updated and thorough investigations on the properties of the most abused NPS in relation with their effects on hair testing. Such investigations are nowadays facilitated by the large availability of adequate analytical instrumentation and validated methods, which are extremely sensitive and accurate at the same time.

From the studies published up to now, a few preliminary conclusions can be tentatively outlined. First, the detection of NPS metabolites appears to be an extremely valuable means to sustain active use and to differentiate active use from passive intake. However, one should be aware that, for some synthetic cannabinoids, alleged metabolites can occasionally be produced also from non-metabolic processes and therefore be detectable in hair even after external contamination. Secondly, the limited data still available from both chronic and sporadic NPS users do not allow to estimate the frequency of use from hair concentrations. However, within homogeneous populations and conditions, large differences in hair concentrations can be interpreted as an effect of use frequency. Third, the scenario of NPS present on the black market is totally unpredictable at any time, in terms of quality and quantity of active principles contained in the finished product. Therefore, it is inappropriate to infer any user's addiction features for NPS from the analytical outcome of hair analysis alone, especially when low concentration levels are detected. As a matter of fact, unaware intake of NPS, sold as surrogates of more traditional drugs, appears to be recurrent, yielding unprecedented hazards of intolerance, adverse effects, and fatal overdose.

## CONFLICT OF INTEREST

The authors have no conflicts of interest to declare

REFERENCES


1. Bossong MG, Van Dijk JP, Niesink RJM. Methylone and mCPP, two new drugs of abuse? Addict Biol. 2005;10(4):321–3.
2. Auwärter V, Dresen S, Weinmann W, Muller M, Putz M, Ferreiros N. "Spice" and other herbal blends: harmless incense or cannabinoid designer drugs? J Mass Spectrom. 2009;44(5):832–7.
3. Kintz P, Salomone A, Vincenti M (Eds). Hair Analysis in Clinical and Forensic Toxicology. Academic Press, 2015.
4. Favretto D, Pascali JP, Tagliaro F. New challenges and innovation in forensic toxicology: focus on the "New Psychoactive Substances". J Chromatogr A 2013; 1287:84–95
5. Pragst F, Balikova M a. State of the art in hair analysis for detection of drug and alcohol abuse. Clin Chim Acta 2006;370(1–2):17–49.
6. Musshoff F, Madea B. Analytical pitfalls in hair testing. Anal Bioanal Chem. 2007;388(7):1475–94.
7. Zawilska JB, Wojcieszak J. Designer cathinones--an emerging class of novel recreational drugs. Forensic Sci Int 2013;231:42–53.
8. Miotto K, Striebel J, Cho AK, Wang C. Clinical and pharmacological aspects of bath salt use: a review of the literature and case reports. Drug Alcohol Depend 2013;132:1–12.
9. Moosmann B, Valcheva S, Neukamm MA, Angerer V, Auwärter V. Hair analysis of synthetic cannabinoids: does the handling of herbal mixtures affect the analyst's hair concentration? Forensic Toxicol. 2015;33:37-44
10. Moosmann B, Roth N, Auwärter V. Finding cannabinoids in hair does not prove cannabis consumption. Sci Rep 2015;5:14906.
11. LeBeau MA, Montgomery MA. Considerations on the utility of hair analysis for cocaine. J Anal Toxicol 2009;33(6):343–4.
12. Bassindale T. Quantitative analysis of methamphetamine in hair of children removed from clandestine laboratories – Evidence of passive exposure? Forensic Sci Int . 2012;219(1–3):179–82.
13. Farst K, Reading Meyer JA, Mac Bird T, James L, Robbins JM. Hair drug testing of children suspected of exposure to the manufacture of methamphetamine. J Forensic Leg Med. 2011;18(3):110–4.
14. Wright J, Kenneally M, Edwards J, Walker S. Evaluation of Environmental methamphetamine exposure through hair analysis. Available from: http://www.tiaft2016.com/program.php

15. Howitt J, Doran G, Deans R, De Filippis C, Kostakis C. Occupational exposure of police officers to illicit drugs – comparing exposure to the outcomes of hair and urine testing. Available from: http://www.tiaft2016.com/program.php
16. Cooper G, Kronstrand R, Kintz P. Society of Hair Testing guidelines for drug testing in hair. Forensic Sci Int 2012;218(1–3):20–4.
17. Salomone A, Tsanaclis L, Agius R, Kintz P, Baumgartner MR. European guidelines for workplace drug and alcohol testing in hair. Drug Test Anal2016 Oct;8(10):996–1004.
18. Shah SB, Deshmukh NIK, Barker J, Petróczi A, Cross P, Archer R, et al. Quantitative analysis of mephedrone using liquid chromatography tandem mass spectroscopy: application to human hair. J Pharm Biomed Anal 2012;61:64–9.
19. Frison G, Frasson S, Zancanaro F, Tedeschi G, Zamengo L. Detection of 3-methylmethcathinone and its metabolites 3-methylephedrine and 3-methylnorephedrine in pubic hair samples by liquid chromatography-high resolution/high accuracy Orbitrap mass spectrometry. Forensic Sci Int 2016;265:131–7. A
20. Kim J, In S, Park Y, Park M, Kim E, Lee S. Deposition of JWH-018, JWH-073 and their metabolites in hair and effect of hair pigmentation. Anal Bioanal Chem 2013;405:9769–78.
21. Kim J, Park Y, Park M, Kim E, Yang W, Baeck S, et al. Simultaneous determination of five naphthoylindole-based synthetic cannabinoids and metabolites and their deposition in human and rat hair. J Pharm Biomed Anal 2015;102:162-175
22. Hutter M, Moosmann B, Kneisel S, Auwärter V. Characteristics of the designer drug and synthetic cannabinoid receptor agonist AM-2201 regarding its chemistry and metabolism. J Mass Spectrom 2013;48:885–94.
23. Auwärter V, Hutter M, Neukamm MA, Moosmann B. O23: Hair analysis for synthetic cannabinoids: How does handling of herbal mixtures during forensic analysis affect the analyst's hair concentrations? Toxicol Anal Clin 2014;26:S14.
24. Hutter M, Moosmann B, Auwarter V, Neukamm MA. Hair analysis for JWH-018, JWH-122, and JWH-210 after passive in vivo exposure to synthetic cannabinoid smoke. Forensic Toxicol. 2015;33(1):69–76.
25. Saito T, Sasaki C, Namera A, Kurihara K, Inokuchi S. Experimental study on external contamination of hair by synthetic cannabinoids and effect of hair treatment. Forensic Toxicol. 2015;33(1):155–8.
26. Park M, Yeon S, Lee J, In S. Determination of XLR-11 and its metabolites in hair by liquid chromatography-tandem mass spectrometry. J Pharm Biomed Anal 2015;114:184–9.

27. Salomone A, Gerace E, Luciano C, DI Corcia D, Vincenti M. O22: Quantification of 22 synthetic cannabinoids and 10 metabolites in human hair. Toxicol Anal Clin 2014;26:S14.
28. Franz F, Angerer V, Hermanns-Clausen M, Auwärter V, Moosmann B. Metabolites of synthetic cannabinoids in hair: proof of consumption or false friends for interpretation? Anal Bioanal Chem 2016;408(13):3445–52.
29. Salomone A, Gerace E, D'Urso F, Di Corcia D, Vincenti M. Simultaneous analysis of several synthetic cannabinoids, THC, CBD and CBN, in hair by ultra-high performance liquid chromatography tandem mass spectrometry. Method validation and application to real samples. J Mass Spectrom 2012;47:604–10.
30. Hutter M, Kneisel S, Auwärter V, Neukamm M. Determination of 22 synthetic cannabinoids in human hair by liquid chromatography-tandem mass spectrometry. J Chromatogr B 2012;903:95–101.
31. Gottardo R, Sorio D, Musile G, Trapani E, Seri C, Serpelloni G, et al. Screening for synthetic cannabinoids in hair by using LC-QTOF MS: A new and powerful approach to study the penetration of these new psychoactive substances in the population. Med Sci Law 2014;54:22–7.
32. Salomone A, Luciano C, Di Corcia D, Gerace E, Vincenti M. Hair analysis as a tool to evaluate the prevalence of synthetic cannabinoids in different populations of drug consumers. Drug Test Anal 2014;6:126–34.
33. Strano-Rossi S, Odoardi S, Fisichella M, Anzillotti L, Gottardo R, Tagliaro F. Screening for new psychoactive substances in hair by ultrahigh performance liquid chromatography-electrospray ionization tandem mass spectrometry. J Chromatogr A 2014;1372C:145–56.
34. Seely K, Patton AL, Moran CL, Womack ML, Prather PL, Fantegrossi WE, et al. Forensic investigation of K2, Spice, and "bath salt" commercial preparations: A three-year study of new designer drug products containing synthetic cannabinoid, stimulant, and hallucinogenic compounds. Forensic Sci Int 2013;233:416–22.
35. Zamengo L, Frison G, Bettin C, Sciarrone R. Understanding the risks associated with the use of new psychoactive substances (NPS): high variability of active ingredients concentration, mislabelled preparations, multiple psychoactive substances in single products. Toxicol Lett 2014;229:220–8.
36. Uchiyama N, Kikura-Hanajiri R, Ogata J, Goda Y. Chemical analysis of synthetic cannabinoids as designer drugs in herbal products. Forensic Sci Int 2010;198(1–3):31–8.

37. Dresen S, Ferreirós N, Pütz M, Westphal F, Zimmermann R, Auwärter V. Monitoring of herbal mixtures potentially containing synthetic cannabinoids as psychoactive compounds. J Mass Spectrom. 2010;45(10):1186–94.
38. Zuba D, Byrska B, Maciow M. Comparison of "herbal highs" composition. Anal Bioanal Chem 2011;400:119–26.
39. Shanks KG, Behonick GS, Dahn T, Terrell A. Identification of Novel Third-Generation Synthetic Cannabinoids in Products by Ultra-Performance Liquid Chromatography and Time-of-Flight Mass Spectrometry. J Anal Toxicol. 2013;(4):517–25.
40. Papanti D, Schifano F, Botteon G, Bertossi F, Mannix J, Vidoni D, et al. "Spiceophrenia": a systematic overview of "spice"-related psychopathological issues and a case report. Hum Psychopharmacol 2013;28:379–89.
41. Vardakou I, Pistos C, Spiliopoulou C. Spice drugs as a new trend: mode of action, identification and legislation. Toxicol Lett 2010;197:157–62.
42. Jang M, Yang W, Jeong S, Park S, Kim J. A fatal case of paramethoxyamphetamine poisoning and its detection in hair. Forensic Sci Int 2016;266:e27–31.
43. Palamar JJ, Salomone A, Vincenti M, Cleland CM. Detection of "bath salts" and other novel psychoactive substances in hair samples of ecstasy/MDMA/"Molly" users. Drug Alcohol Depend 2016;161:200–5.
44. Salomone A, Gazzilli G, Di Corcia D, Gerace E, Vincenti M. Determination of cathinones and other stimulant, psychedelic, and dissociative designer drugs in real hair samples. Anal Bioanal Chem 2016;408(8):2035–42.
45. Martin M, Muller JF, Turner K, Duez M, Cirimele V. Evidence of mephedrone chronic abuse through hair analysis using GC/MS. Forensic Sci Int 2012;218:44–8.
46. Gerace E, Petrarulo M, Bison F, Salomone A, Vincenti M. Toxicological findings in a fatal multidrug intoxication involving mephedrone. Forensic Sci Int 2014;243C:68–73.
47. Wikström M, Thelander G, Nyström I, Kronstrand R. Two Fatal Intoxications with the New Designer Drug Methedrone ( 4-Methoxymethcathinone ) Autopsy cases. J Anal Toxicol. 2010;34:594–8.
48. Alvarez J-C, Etting I, Abe E, Villa A, N, Knapp A, Fabresse N. Identification and quantification of 4-methylethcathinone (4-MEC) and 3,4-methylenedioxypyrovalerone (MDPV) in hair by LC–MS/MS after chronic administration. Forensic Sci Int 2017;270:39–45.

49. Alvarez J-C, Fabresse N, Knapp A, El Hajj Sleiman I, Garnier R, Langrand J. Identification and quantification of diphenidine in hair by LC-MS/MS after single administration. Toxicol Anal Clin 2016; doi 10.1016/j.toxac.2016.09.006
50. Gerace E, Bovetto E, Di Corcia D, Vincenti M, Salomone A. A case of non-fatal intoxication associated with the recreational use of diphenidine, J. Forensic Sci, 2016, DOI: 10.1111/1556-4029.13355
51. Namera A, Konuma K, Saito T, Ota S, Oikawa H, Miyazaki S, et al. Simple segmental hair analysis for α-pyrrolidinophenone-type designer drugs by MonoSpin extraction for evaluation of abuse history. J Chromatogr B A 2013;942–943:15–20.
52. Namera A, Urabe S, Saito T, Torikoshi-Hatano A, Shiraishi H, Arima Y, et al. A fatal case of 3,4-methylenedioxypyrovalerone poisoning: coexistence of α-pyrrolidinobutiophenone and α-pyrrolidinovalerophenone in blood and/or hair. Forensic Toxicol 2013;31:338–43.
53. Nieddu M, Burrai L, Demontis MP, Varoni MV, Baralla E, Trignano C, et al. Simultaneous determination of 11 illicit phenethylamines in hair by LC-MS-MS: In vivo application. J Anal Toxicol. 2015;39(7):532–7.
54. Cirimele V, Klinger N, Etter M, Duez M, Humbert L, Gaulier J-M, et al. O21: Testing for 18 synthetic cannabinoids in hair using HPLC-MS/MS: Method development and validation, its application to authentic samples and preliminary results. Toxicol Anal Clin 2014;26:S13.
55. Schaefer N, Peters B, Bregel D, Kneisel S, Schmidt PH, Ewald AH. A fatal case involving several synthetic cannabinoids. Toxichem Krimtech 2013;80:248–51.

Centro Regionale Antidoping
Alessandro Bertinaria
LABORATORIO REGIONALE DI TOSSICOLOGIA

Torino, December 22nd, 2016

**Dr. Pascal Kintz**
Editor in Chief
**Toxicologie Analytique et Clinique**

Dear Editor,

I am pleased to submit the reviewed version of the manuscript n. TOXAC-S-16-0068 entitled "Interpretation of NPS results in real hair samples" for publication on Toxicologie Analytique et Clinique.

The manuscript was reviewed following all the Referees' comments and recommendations. Two new references (#15 and #48 were added) and one was deleted (former #20), bringing up the new total to 55 items.

All the revisions in the text and Tables were marked using a yellow highlighting. The answers to the Referees' comments are listed below:

**Reviewer #1 comments:**

1. In this review a total of 55 papers was presented. Among these, it would be hard to identify which results are to be included in the abstract. Post-mortem cases were discussed through the text
2. Text was modified. Ref. 11 describes the risk for police officers to be contaminated. Ref. 15 was also added
3. The issue of setting a cut-off is discussed in the paragraph "SPORADIC OR CHRONIC USE?" and in the Conclusions, which was further extended
4. We thank the referee for his interest in our work. Our experience is described in the text and our publications are cited (refs 27, 29, 32, 43, 44, 46, 49). Our recommendations concerning passive exposure, unaware intake and chronic or sporadic use are presented in the Conclusions
5. Comments were added in the Introduction

**Reviewer #2 comments:**

Major point

The text was modified in order to acknowledge that in some cases also metabolites can be present merely after passive contamination

Minor points

Page 4, line 4: false was replaced with misleading

Line 25: absolutely true. The fact that also non-cathinones compounds were grouped for the sake of simplicity under the definition “synthetic cathinones” was highlighted in the text

Line 26: subset was corrected

Line 33-35: the text was modified

Page 5, line 3: the text was clarified

Line 7/8: the text was modified

Line 16: the result was originally reported as shown in the cited paper. However, text was modified as recommended by the reviewer

Line 19: i.e. stands for “namely”. The text was modified in the attempt of making it more clear

Line 34: the concentration was deleted

Page 6: lines and reference were deleted

Page 7, lines 10-12: the text was modified

Line 14: the references were updated in the text

Line 15: the text was modified

Page 8, line 11: the text was modified

Page 9, line 5: the text was modified

Page 10, lines 3-4: the sentence was modified

Page 10, line 26: the heading was modified as suggested

Page 11, line 15: Missing information was added

Line 19: named was omitted as suggested

Line 22: we decided to report the explanation provided by the authors

Page 12, line 1: sentence was modified in the attempt of clarifying it

Line 7: more information about the segmentation was added. Unfortunately, concentration in ng/mg is not available

Line 8: the text was clarified

Line 10: the authors refereed to cut-off for amphetamines. Text was modified

Line 16: to was omitted as suggested

Line 17: the text was modified as suggested

Page 13: the Discussion was expanded