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RESEARCH ARTICLE

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Prevalence and concentrations of new designer stimulants, synthetic opioids, benzodiazepines, and hallucinogens in postmortem hair samples: A 13-year retrospective study

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Abstract

Hair samples are frequently analyzed in order to characterize consumption patterns of drugs. However, the interpretation of new psychoactive substance (NPS) findings in hair remains difficult because of lacking data for comparison. In this study, selected postmortem hair samples ($n = 1203$) from 2008 to 2020 were reanalyzed for synthetic cathinones, piperazines, phenethylamines, hallucinogens, benzodiazepines and opioids to evaluate prevalence data and concentration ranges. Hair samples were extracted using a two-step extraction procedure and analyzed using a validated liquid chromatography–tandem mass spectrometry (LC–MS/MS) method. Overall NPSs were detected in 381 cases (31.6%). Many cases were tested positive for more than one NPS in the same time span. A variety of NPS with a large range of concentrations was observed. For better comparability and interpretation of positive cases in routine work, quantitation data for 13 NPS were calculated as percentiles. The most frequently detected NPS in this study were *N*-ethylamphetamine, α-pyrrolidinovalerophenone, mephedrone, benzedrone, metamfepramone, and 4-fluoroamphetamine. In conclusion, a high prevalence of these drugs was observed from postmortem hair samples. The results show a growing use of many different NPSs by mainly young drug-using adults. Consequently, NPS screening procedures should be included in forensic toxicology. Our quantitative data may support other toxicologists in their assessment of NPS hair concentrations.

KEYWORDS
hair concentrations, LC–MS/MS, new psychoactive substances (NPS), postmortem hair analysis, prevalence data

1 | INTRODUCTION

The diffusion of legal highs or new psychoactive substances (NPS) in the illicit drug market is a worldwide phenomenon. A characteristic feature of these mostly synthetic substances is their similarity to controlled drugs of abuse in terms of effect and/or chemical structure.[1] It has been shown that some of these substances are extensively more potent than their analogues. Several cases of acute toxicity and deaths

have been already reported to this novel class of compounds.[2–4] NPSs are mostly sold on the internet or in so-called "head shops" as "plant food," "bath salts," or as "research chemicals" and are strictly marked "not for human consumption."[5]

To eliminate or at least reduce the problem of the uncontrolled sale of NPSs, different legal approaches have been applied. Similar to most European countries, in Germany, drug law was based on the resolutions of the United Nations, which means the ban of specific target analytes. This system of individual drug listing has been exploited by producers of legal highs. Because a lot of confiscated preparations contained a new substance, this substance was included in the list of controlled substances. However, the ban of one substance resulted in the marketing of its noncontrolled derivatives or new substances.[1] Therefore, in Germany, the "New Psychoactive Substances Act" came into force on November 26, 2016.[6] It supplements the individual substance approach of the drug law by containing a substance group regulation so that a large number of isomers and structurally similar compounds are also prohibited. The aim of this substance group regulation is to counter NPS more effectively in legal terms and to combat the distribution and availability, the purchase as well as the production of these substances.[6]

So far, research on NPS has mostly been limited to different case reports,[3–7] self-reported use in user surveys,[8] measurement of wastewater,[9,10] reports on seized substances,[1] analysis of pooled urine from portable urinals,[11,12] and poison center data.[13] However, there are currently little scientific data on the prevalence of NPS in Europe, especially in Germany. Many of these data are based on questionnaire surveys and only a few on analyses of biological matrices.[14–16] One difficulty in detecting NPS is that they usually cannot be detected by simple screening tests because these substances do not interact with common immunoassays.[17] Furthermore, the detection and identification process of NPS is not easy because the chemical, chromatographic, and spectral properties of many substances are very similar.[1] In addition, NPS are not often in the requested portfolio for the analyzing laboratories, due to the lack of reference standards. Therefore, a high number of undetected cases can be assumed.

Hair analysis provides an overview of the drug consumption behavior over a wide detection window, ranging from weeks to several months according to hair shaft's length.[18] Therefore, hair seems to be an appropriate sample matrix for retrospective prevalence studies and to investigate NPS-related history. Hair samples are frequently analyzed in order to characterize consumption patterns of drugs of abuse. However, the interpretation of NPS-positive hair samples remains difficult because of lacking data for comparison.

The aim of this study was to extend a previously published LC–MS/MS method for the quantification of synthetic cathinones and piperazines in hair[19] with other NPS from different subclasses (e.g., 2C-X substances, amphetamines, hallucinogens, opioids, and benzodiazepines) and to validate the method. Moreover, selected hair samples ($n = 1203$) from 2008 to 2020 were reanalyzed for these NPS to evaluate prevalence data and concentration ranges in postmortem cases.

2 | MATERIALS AND METHODS

2.1 | Reagents and chemicals

Reference standards including buphedrone, bupropion, butylone, benzylpiperazine (BZP), 4-bromo-2,5-dimethoxyphenethylamine (2C-B), 2-(8-bromo-2,3,6,7-tetrahydrobenzo[1,2-b:4,5-b′]difuran-4-yl) ethan-1-amine (2C-B-FLY), 1-(8-bromobenzo[1,2-b;4,5-b′]difuran-4-yl)-2-aminopropane (Bromo-DragonFLY), cathinone, 4-chloro-2,-5-dimethoxyphenethylamine (2C-C), meta-chlorphenylpiperazine (mCPP), dibutylone, dimethyltryptamine (DMT), *N*-ethylamphetamine, ethylcathinone, ethylone, etizolam, eutylone, 4-fluoromethcathinone (4-FMC), heliomethyamine, 3′,4′-methylenedioxy-α-pyrrolidinopropiophenone (MDPPP), mephedrone (4-MMC), mescaline, methcathinone, p-methoxyamphetamine (PMA), 4-methoxyphencyclidine (4-MeO-PCP), methylbuphedrone, methylenedioxypyrovalerone (MDPV), 4-methylethcathinone (4-MEC), methylone, 1-methyl-4-phenyl-4-propionoxypiperidine (MPPP), 4-methyl-α-pyrrolidinohexiophenone (MPHP), naphyrone, pentylone, phencyclidine (PCP), pyrazolam, α-pyrrolidinobutiophenone (α-PBP), α-pyrrolidinopropiophenone (α-PPP), α-pyrrolidinovalerophenone (α-PVP), trazodone, 3-trifluormethylphenylpiperazine (3-TFMPP), U-47700, and internal standards (7-aminoclonazepam-D4, 7-aminoflunitrazepam-D7, amphetamine-D5, buprenorphine-D4, cocaethylen-D3, cocaine-D3, fentanyl-D5, ketamin-D4, MDMA-D5, MDE-D6, methadone-D9, methamphetamine-D5, nortilidin-D3, tilidin-D6, and tramadol-D3) were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). Deschloroketamine (O-PCM), ethyldeschloroketamine (2-oxo-PCE) were obtained from Cayman Chemical Company (Ann Arbor, USA), while 4-fluoroamphetamine (4-FA) were purchased from Lipomed (Weil am Rhein, Germany) and amfepramone from Toronto Research Chemicals (North York, Canada). Benzedrone, brephedrone (4-BMC), brotizolam, 3,4-dimethylmethcathinone (3,4-DMMC), ethylbuphedrone (NEB), metamfepramone, methedrone, 5-methoxydimethyltryptamine (5-MeO-DMT), para-methoxyphenylpiperazine (MeOPP), 4-methoxy-α-pyrrolidinopropiophenone (MOPPP), 4-methyl-α-pyrrolidinobutiophenone (MPBP), pentedrone, and pyrovalerone were purchased from LGC Standards (Wesel, Germany)

In some of the fatal cases, drug samples (capsules, powders, or tablets) were available. These substances (clonazolam, delorazepam, diclazepam, flubromazepam, p-fluorofentanyl, 1-[1-benzofuran-2-yl]-*N*-methylpropan-2-amine [2-MAPB], metizolam, ocfentanil, and phenazepam) were also used as reference after dissolution and dilution, but were no part of full method validation and were only taken for identification.

Liquid chromatography–mass spectrometry (LC–MS) grade methanol and acetonitrile were obtained by Fisher Scientific GmbH (Schwerte, Germany), ammonium formate (LC–MS grade) from Agilent Technologies and formic acid (purity of >99%) from Acros Organics (Geel, Belgium). High-performance liquid chromatography (HPLC) grade water was obtained from an ELGA PureLab flex purification system (Celle, Germany).

2.2 | LC–MS/MS apparatus and characteristics

The liquid chromatography–tandem mass spectrometry (LC–MS/MS) system consisted of a 6460 triple quadrupole mass spectrometer (Agilent Technologies, Waldbronn, Germany) and an electrospray-ionization (ESI) Agilent Jet Stream source operated in positive ionization mode. The liquid chromatography system consisted of an LC infinity 1290 (Agilent Technologies, Waldbronn, Germany). Liquid chromatography was performed on a Kinetex C18 column (2.1 × 150 mm, 1.7 μm, Phenomenex, Torrance, USA). By gradient elution (water with 0.1% formic acid [Eluent A] and acetonitrile [Eluent B]), an evenly spread elution over a runtime of 11 min was obtained. The method was programmed as follows: 0–1 min 12% B; 1–4.5 min 13% B; 4.5–6.5 min 15% B; 6.5–9 min 60% B; 9–10 min 95% B; and from 10–11 min there was a linear decrease to 12% B (starting conditions). The injection volume was 5 μl and the flow rate was 0.4 ml/min. The temperature of the column was set at 40°C.

Source parameters had a drying gas (nitrogen) temperature of 210°C, drying gas flow of 10 L/min, nebulizer pressure of 45 psi and a capillary voltage of 3000 V in positive mode. The application of a dynamic multiple reaction monitoring (dMRM) mode provided further optimization of sensitivity, reproducibility, and precision. At least two MRM transitions were selected for each analyte to achieve sufficient selectivity. The analyte-specific LC–MS/MS parameters are listed in Table S1.

For the method validation, sample measurements and quantitation, the data were analyzed using MassHunter Workstation Software version B.06.00, MassHunter Qualitative Analysis (B.06.00), MassHunter Quantitative Analysis (B.06.00) and Valistat 2.0 Arvecon GmbH (Walldorf, Germany). Percentiles were calculated with SPSS 27.0 (IBM, USA). All figures were created using Microsoft Excel 2016 (Microsoft, USA).

2.3 | Sample collection

Forensic autopsies are performed in Germany's state capital Berlin (which has a population of about 3.5 million inhabitants) at the authors' Institute of Forensic Medicine at the Charité—Universitätsmedizin Berlin and the Governmental Institute of Legal Medicine and Forensic Sciences. In the period from 2008 to 2020, 13,494 autopsies were done at the authors' institute. In about 80% of the cases, a toxicological analysis was ordered and, if available, corresponding samples (blood, urine, hair, stomach content, and organ samples) were asserted. Unfortunately, hair was not available for all fatalities.

In total, 1203 hair samples were reanalyzed concerning NPSs. Samples were selected due to the age (samples from deceased persons up to 60 years were included) and case history, which meant a suspected or proven use of common drugs of abuse (e.g., amphetamines, cocaine, opiates/opioids, cannabinoids, and benzodiazepines). The limits of detection (LODs) of the analytical method were used as the minimum criterion to identify positive samples.

Hair samples were taken before autopsy to avoid a possible contamination with body fluids. Head and body hairs were bundled together at the proximal end so that the end close to the skin was visible. Hair lengths longer than 6 cm were shortened and only the area close to the skin (0–6 cm) was investigated. Shorter hairs were examined in full length.

2.4 | Sample preparation

Hair sample preparation was done as published in detail before.[20] To minimize external contamination, hair samples were washed prior to extraction with water and acetone. Each of the cleaned and dried hair samples were cut into small snippets, and 50 mg was transferred into an extraction vial. Aliquots of 500 μl extraction buffer (methanol/acetonitrile/H_2O with ammonium formate; 25/25/50) and 5 μl of the internal standard mix (1 ng/μl) were added. The samples were shaken twice at 40°C for 18 h at 900 rpm. The supernatant of the first extraction was transferred into another vial and stored at −20°C in the freezer, while for the second extraction another 500 μl of extraction buffer was added to the hair sample. Both supernatants were combined and filtered through a regenerated cellulose (RC) membrane filter (Ø = 4 mm, 0.2-μm pore size, Phenomenex) into a vial for measurement. If the measured concentrations were above the highest calibration point, the final hair sample extracts were diluted and reinjected into the LC–MS/MS system.

2.5 | Method validation

The method was validated according to the guidelines of the Society of Toxicological and Forensic Chemistry (GTFCh).[21] As previously published in detail,[19] this includes different test parameters (selectivity, linearity, LOD and quantitation, accuracy, analyte stability, recovery and matrix effect). At first, the selectivity was confirmed by analyzing six different blank hair samples, once with and once without the addition of the internal standard mixture. Furthermore, negative hair samples were spiked with common drugs of abuse that may be expected in authentic samples. For the calibration, 50 mg of negative hair sample were spiked with concentrations ranging from 0.01 to 3 ng/mg (calibration levels: 0.01 ng/mg, 0.025 ng/mg, 0.05 ng/mg, 0.1 ng/mg, 0.25 ng/mg, 0.5 ng/mg, 0.75 ng/mg, 1 ng/mg, 2 ng/mg, 3 ng/mg). The calibration range varied for the different analytes. For each calibration level ($n = 10$), six separately prepared samples were analyzed. The area ratios of analyte versus internal standard were plotted to a function of substance concentration. The LOD and lower limit of quantification (LLOQ) were estimated according to DIN 32645 by spiking at least six hair samples from the lower end of the calibration curve (calibration range: 0.02 to 0.12 ng/mg).[22] The accuracy of the method was confirmed daily over a period of 5 days by analyzing blank hair samples, which had been spiked with concentrations ranging from 0.06 to 0.6 ng/mg. The bias, repeatability, and intermediate precision were estimated. The stability of the analytes in

TABLE 1 Validation parameters I (linear range, curve equation, weighting factor, correlation coefficient [r^2], and analytical limits)

	Calibration curve				Analytical limits	
Analytes	Linear range (ng/mg)	Curve equation	r^2	Weighting factor	LOD (ng/mg)	LLOQ (ng/mg)
2C-B	0.025–1	$y = 14.542872x + 0.002380$	0.993	$1/x^2$	0.009	0.011
2C-B-FLY	0.025–1	$y = 9.649704x + 6.240688 \times 10^{-7}$	0.994	$1/x^2$	0.014	0.016
2C-C	0.025–1	$y = 66.630241x + 0.091981$	0.995	$1/x^2$	0.005	0.016
2-Oxo-PCE	0.01–2	$y = 330.581099x + 0.087601$	0.999	$1/x$	0.006	0.010
3,4-DMMC	0.01–3	$y = 93.393040x + 0.006929$	0.997	$1/x^2$	0.005	0.009
3-TFMPP	0.01–1	$y = 12.294494x + 0.010749$	0.995	$1/x$	0.004	0.009
4-FA	0.01–3	$y = 1.603579x + 0.002876$	0.998	$1/x$	0.005	0.007
4-FMC	0.025–3	$y = 73.675090x + 3.955095 \times 10^{-4}$	0.997	$1/x^2$	0.003	0.011
4-MEC	0.01–3	$y = 472.349542x + 0.127985$	0.997	$1/x^2$	0.006	0.010
4-MeO-PCP	0.025–2	$y = 145.968434x + 0.054435$	0.993	$1/x^2$	0.009	0.024
5-MeO-DMT	0.01–1	$y = 67.691424x + 0.009447$	0.997	$1/x$	0.005	0.010
Amfepramone	0.025–3	$y = 26.613206x - 0.003054$	0.999	$1/x$	0.011	0.013
Benzedrone	0.01–1	$y = 33.369925x + 0.012627$	0.994	$1/x^2$	0.005	0.008
Benzylpiperazine	0.025–3	$y = 10.442321x - 0.017812$	0.992	$1/x^2$	0.009	0.018
Brephedrone	0.01–1	$y = 139.927115x + 0.036569$	0.996	$1/x$	0.006	0.008
Bromo-DragonFLY	0.025–1	$y = 6.926570x - 0.001155$	0.992	$1/x^2$	0.008	0.013
Brotizolam	0.025–0.75	$y = 2.197328x + 0.010542$	0.992	$1/x^2$	0.012	0.023
Buphedrone	0.025–3	$y = 15.979170x - 0.001784$	0.998	$1/x^2$	0.004	0.014
Bupropion	0.01–1	$y = 57.596302x + 0.014299$	0.997	$1/x^2$	0.003	0.009
Butylone	0.025–3	$y = 40.742731x + 0.004938$	0.997	$1/x^2$	0.008	0.013
Cathinone	0.025–3	$y = 1.119522x + 0.036873$	0.997	$1/x^2$	0.009	0.016
mCPP	0.01–1	$y = 8.537957x - 5.192308 \times 110^{-4}$	0.999	$1/x^2$	0.005	0.009
Deschloroketamine	0.01–3	$y = 34.869043x + 0.003312$	0.997	$1/x^2$	0.006	0.009
Dibutylone	0.025–3	$y = 39.634210x + 0.010390$	0.997	$1/x^2$	0.007	0.013
DMT	0.01–2	$y = 57.668281x - 0.002043$	0.997	$1/x^2$	0.006	0.008
N-Ethylamphetamine	0.01–3	$y = 75.953680x + 0.097463$	0.994	$1/x^2$	0.003	0.007
Ethylcathinone	0.01–3	$y = 74.364994x - 0.057161$	0.998	$1/x$	0.007	0.010
Ethylone	0.01–3	$y = 52.529384x + 0.015791$	0.998	$1/x$	0.006	0.010
Etizolam	0.025–0.75	$y = 5.736533x + 0.004885$	0.993	$1/x$	0.013	0.024
Eutylone	0.025–3	$y = 107.601221x + 0.025287$	0.994	$1/x^2$	0.009	0.016
Heliomethylamine	0.01–3	$y = 415.374718x + 0.027428$	0.996	$1/x^2$	0.004	0.009
MDPPP	0.025–3	$y = 39.990247x - 0.003727$	0.994	$1/x^2$	0.003	0.017
MDPV	0.01–3	$y = 15.878661x + 0.003642$	0.997	$1/x^2$	0.006	0.008
MeOPP	0.025–3	$y = 14.970140x - 0.006598$	0.994	$1/x^2$	0.005	0.012
Mephedrone	0.01–3	$y = 205.165545x + 0.066257$	0.998	$1/x^2$	0.006	0.008
Mescaline	0.025–3	$y = 48.215146x + 0.008302$	0.994	$1/x^2$	0.008	0.015
Metamfepramone	0.01–3	$y = 63.191169x + 0.005467$	0.996	$1/x^2$	0.005	0.009
Methcathinone	0.01–3	$y = 34.908263x + 0.010261$	0.995	$1/x^2$	0.006	0.008
methedrone	0.025–3	$y = 123.474408x - 0.010638$	0.993	$1/x^2$	0.003	0.015
methylbuphedrone	0.025–3	$y = 66.588181x + 0.039809$	0.996	$1/x$	0.005	0.015
methylone	0.025–3	$y = 52.772469x + 0.682200$	0.995	$1/x^2$	0.004	0.014
MOPPP	0.025–3	$y = 91.021195x + 0.001997$	0.997	$1/x^2$	0.005	0.014
MPBP	0.01–3	$y = 23.698691x - 0.022596$	0.998	$1/x$	0.004	0.005
MPHP	0.025–3	$y = 1405.311781x - 0.199823$	0.996	$1/x^2$	0.009	0.017

(Continues)

TABLE 1 (Continued)

Analytes	Calibration curve: Linear range (ng/mg)	Curve equation	r^2	Weighting factor	Analytical limits: LOD (ng/mg)	LLOQ (ng/mg)
MPPP	0.025–3	$y = 28.766555x + 0.002812$	0.996	$1/x^2$	0.007	0.013
Naphyrone	0.025–3	$y = 1642.905400x - 0.082828$	0.995	$1/x^2$	0.005	0.013
NEB	0.025–3	$y = 39.185276x + 0.0328829$	0.998	$1/x^2$	0.007	0.012
α-PBP	0.025–3	$y = 66.033017x - 0.005791$	0.998	$1/x^2$	0.003	0.012
PCP	0.025–2	$y = 70.359583x - 0.001053$	0.996	$1/x^2$	0.004	0.011
Pentedrone	0.025–3	$y = 171.866081x + 0.009665$	0.994	$1/x^2$	0.007	0.013
Pentylone	0.025–3	$y = 99.437251x + 0.114648$	0.994	$1/x^2$	0.007	0.016
PMA	0.05–1	$y = 37.734531x + 3.346126$	0.993	$1/x^2$	0.008	0.019
α-PPP	0.025–3	$y = 53.807225x - 0.028702$	0.999	$1/x^2$	0.003	0.008
α-PVP	0.025–3	$y = 32.388852x + 0.001594$	0.994	$1/x^2$	0.008	0.012
Pyrazolam	0.025–1	$y = 1.196023x + 8.765157 - 10^{-4}$	0.995	$1/x$	0.008	0.021
Pyrovalerone	0.025–3	$y = 29.587548x - 0.078014$	0.999	$1/x^2$	0.008	0.014
Trazodone	0.025–1	$y = 37.475030x + 0.065257$	0.996	$1/x$	0.009	0.015
U-47700	0.01–3	$y = 12.459543x + 0.019787$	0.997	$1/x^2$	0.007	0.009

the hair samples (stored in the autosampler tray at 5°C) was also tested with the spiked hair extracts. Six spiked hair samples at concentrations of between 0.06 and 0.6 ng/mg were prepared, pooled, and divided into six aliquots, and then the peak areas were measured. The following time intervals were chosen: 0, 24, 48, and 72 h. Recovery and possible matrix effects were determined according to Matuszewski et al.[23] by measuring the high and low concentrations (0.06 and 0.6 ng/mg) in five different hair sample matrices.

3 | RESULTS

3.1 | Method validation

No coeluting or interfering substances were observed in the chromatogram of the different blank hair samples, which is an indicator for the selectivity of the method. The results of the linear calibration were checked for outliers using the Grubbs' test at a 99% significance level. The homogeneity of variance was confirmed by the Cochran test. Linearity was shown by applying Mandel's *F* test. For each of the 58 analytes, linearity was confirmed over the calibration range with correlation coefficient values varying between 0.992 and 0.999. As shown in Table 1, $1/\times$ or $1/\times^2$ weighted linear regression was used for all compounds. The LOD varied from 3 to 14 pg/mg, while the LOQ ranged from 5 to 24 pg/mg (Table 1).

Accuracy data for all analytes were within the acceptance range as proposed in the guidelines of the GTFCh. The results are summarized in Table S2. Despite a minimal loss of substance (<25%), sufficient stability of the analytes was confirmed over a period of 72 h at 5°C. The matrix effect was acceptable, ranging from 77.0% to 109.6% (Table S2). In addition, the recovery was estimated to be over 60% for all analytes.

3.2 | NPS findings in postmortem hair samples

From the period between 2008 and 2020, 1203 postmortem hair samples were reanalyzed. Overall, NPSs were detected in 381 cases. For determining the prevalence, results from all hair colors (from blonde to black), from parts of the body (chest and pubic hair), and from cosmetically treated specimens were included, even if we are aware of the limitations. The length of the analyzed proximal hair segments varied between 0.5 and 6 cm.

The age range for the cases under study was 14–60 years (mean 35.1 years; median 34.5 years). In 14 cases the age was unknown. Among the deceased, 77% (930 cases) were male, and 273 cases were female (23%). Positive specimens were not equally distributed between males ($n = 299$; 78%) and females ($n = 82$; 22%). The highest number of positive cases ($n = 73$, 19%) was observed in the 31 to 35 age group (Figure 1).

The prevalence of NPS in postmortem hair samples was 31.6%. Among all NPS positive cases, 107 hair samples were positive for two or more (up to 10) NPS. During the 13-year period, 48 different target analytes were identified in positive cases. The NPS found can be grouped into the following subclasses: cathinones (47.1%), amphetamines (24.1%), piperazines (10.9%), arylcyclohexylamines (6.9%), 2C-X substances (3.4%), benzodiazepines (3.0%), and other compounds (4.6%). The frequency of NPS detected in postmortem hair samples per year between January 2008 and December 2020 are summarized in detail in Figure 2 and Table 2. As mCPP is formed during metabolism of the antidepressant trazodone, trazodone was included in this study in order to distinguish between a trazodone treatment and a direct mCPP intake. Indeed, 12 mCPP positive hair samples were also positive for trazodone. Table 2 shows only those mCPP cases ($n = 17$) where no trazodone was detected.

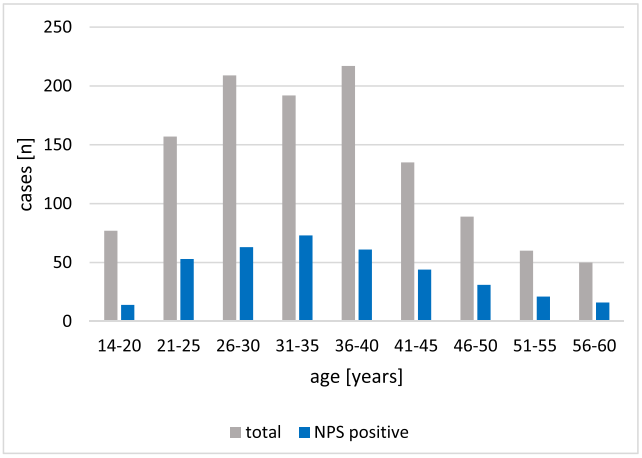

FIGURE 1 Age distribution of total and new psychoactive substance (NPS) positive cases [Colour figure can be viewed at wileyonlinelibrary.com]

The most frequently detected NPS was *N*-ethylamphetamine, which was found in 81 hair samples. A clear increase in frequency was observed from 2008 to 2020. While 4-FA and mCPP were the most frequent NPS up to 2011, *N*-ethylamphetamine took top spot in 2018 and 2020.

Other multiple detections included α-PVP, mephedrone, benzedrone, metamfepramone, and 4-FA. For 4-FA, there was a decrease in case frequency from 2009 to 2020, with no positive detection between 2015 and 2019. In contrast, α-PVP could always be detected over the whole time period, as well as nearly always benzedrone, metamfepramone, and mephedrone, with a detection peak in 2017, 2014, 2015, and 2019, respectively. MDPV was detected, with the highest number of cases ($n = 6$), first in 2011 and showed a decreasing trend until today. In the piperazine group, 3-TFMPP were found in 24 cases, mCPP in 17 cases, BZP in 6 cases, and MeOPP in only 4 cases. 3-TFMPP were mostly detected in 2013 ($n = 5$) and mCPP in 2009 ($n = 6$).

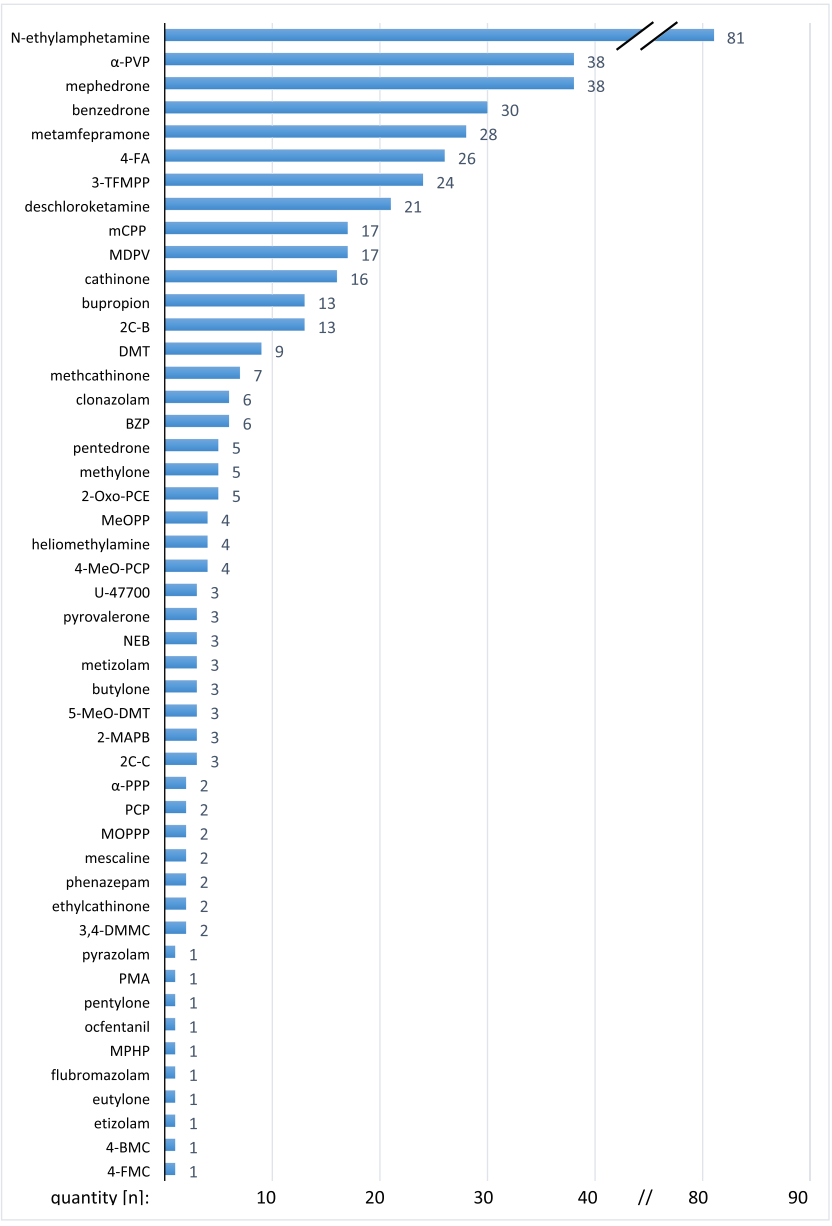

FIGURE 2 Total number of detected new psychoactive substance (NPS) in postmortem hair samples [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Frequency of detected new psychoactive substances (NPS) in postmortem hair samples per year (2008–2020)

	NPS findings per year												
NPS	**2008**	**2009**	**2010**	**2011**	**2012**	**2013**	**2014**	**2015**	**2016**	**2017**	**2018**	**2019**	**2020**
2C-B							1		1		4	4	3
2C-C				1			1		1				
2-MAPB	1						1						1
2-Oxo-PCE						1		1					3
3,4-DMMC								2					
3-TFMPP	1			2	2	5	2	1	2	3		3	3
4-FA		6	3	6	3	5	1						2
4-FMC								1					
4-MeO-PCP												1	3
5-MeO-DMT												1	2
Benzedrone	1		2	2	3	1	6	3		4	3	3	2
Benzylpiperazine							2		3			1	
Brephedrone								1					
Bupropion	2		1	1			1		1	2		2	3
Butylone				1							1	1	
Cathinone	3	3		1				1		1	4	2	1
Clonazolam	1							1	1	2			1
mCPP	5	6	1	3	1	1							
Deschloroketamine			1		1		1	5	2	1	2	2	6
DMT									1			3	5
N-Ethylamphetamine	1	1		2	1	4	4	7	14	8	15	9	15
Ethylcathinone										1			1
Etizolam													1
Eutylone												1	
Flubromazolam													1
Heliomethylamine				1				2			1		
MDPV				6	2	3	1	2		1	1		1
MeOPP			1	1	1	1							
mephedrone		2	1		1	2	1	2	4	6	4	8	7
mescaline			1									1	
Metamfepramone	2	4	1	2		1	2	5	2	3	3	3	
Methcathinone			1					1	4				1
Methylone						3	1		1				
Metizolam									1		1		1
MOPPP							1					1	
MPHP												1	
NEB				1						1			1
Ocfentanil										1			
PCP						1					1		
Pentedrone				2			1	1	1				
Pentylone												1	
Phenazepam		1		1									
PMA				1									
α-PPP	2												
α-PVP	1	1	2	1	2	4	4	2	4	7	1	6	3

TABLE 2 (Continued)

	NPS findings per year												
NPS	**2008**	**2009**	**2010**	**2011**	**2012**	**2013**	**2014**	**2015**	**2016**	**2017**	**2018**	**2019**	**2020**
Pyrazolam								1					
Pyrovalerone		1						1	1				
U-47700										2			1
NPS positive cases	**20**	**25**	**15**	**35**	**17**	**32**	**31**	**40**	**44**	**43**	**41**	**54**	**68**
Total case number	**51**	**68**	**49**	**75**	**81**	**108**	**116**	**88**	**99**	**96**	**106**	**129**	**137**

TABLE 3 Percentile plot of new psychoactive substance (NPS) concentrations found in postmortem hair samples

			Hair concentrations (ng/mg) in percentiles						
NPS	***n***	**Min.**	**10th**	**25th**	**50th**	**75th**	**90th**	**95th**	**Max.**
2C-B	7	0.0143	0.0143	0.0164	0.0237	0.0605	0.121	0.121	0.121
3-TFMPP	13	0.0103	0.0106	0.0123	0.0188	0.0421	0.0794	0.099	0.099
4-FA	24	0.011	0.0135	0.0501	0.0967	1.10	48.5	107.3	113.6
Benzedrone	7	0.0112	0.0112	0.0124	0.0381	0.053	0.152	0.152	0.152
Benzylpiperazine	5	0.0784	0.0784	0.0864	0.098	0.155	0.196	0.196	0.196
Bupropion	6	0.0119	0.0119	0.0344	0.126	1.06	3.47	3.47	3.47
Cathinone	8	0.0198	0.0198	0.0392	0.247	0.43	1.27	1.27	1.27
mCPP	10	0.012	0.0121	0.0368	0.0954	0.228	0.553	0.58	0.58
DMT	6	0.0133	0.0133	0.0142	0.221	0.530	0.609	0.609	0.609
N-Ethylamphetamine	26	0.0101	0.0104	0.0141	0.0242	0.167	0.5001	0.588	0.633
MDPV	10	0.0158	0.0159	0.0228	0.0372	0.569	7.99	8.79	8.79
Mephedrone	14	0.0108	0.0117	0.0313	0.0493	1.15	2.56	3.50	3.50
Metamfepramone	19	0.0103	0.0116	0.0149	0.0297	0.0411	0.0608	0.0721	0.0721

Compared with 2C-C, which was already detected in 2011, 2C-B was first seen in 2014 with most positive cases ($n = 4$) in 2018 and 2019. Table 2 further illustrates that many substances were only detected sporadically ($n \leq 5$) during the total study period, such as 2C-C, 2-MAPB, 2-Oxo-PCE, 3,4-DMMC, 4-FMC, 4-MeO-PCP, 5-MeO-DMT, brephedrone, butylone, ethylcathinone, etizolam, eutylone, flubromazolam, heliomethylamine, mescaline, methylone, metizolam, MOPPP, MPHP, NEB, ocfentanyl, PCP, pentedrone, pentylone, PMA, α-PPP, pyrazolam, pyrovalerone, and U-47700. Of the specific NPS detected, α-PPP were only found in casework in 2008, whereas PMA were only found in 2011, ocfentanyl in 2017, 3,4-DMMC, flephedrone (4-FMC), brephedrone and pyrazolam in 2015, eutylone, MPHP, and pentylone in 2019 and etizolam and flubromazolam in 2020. However, 68 NPSs were detected solely in 2020, showing an increase in numbers compared with 2008 ($n = 20$).

Out of the 381 positive NPS hair samples, concentrations above the analyte-specific limit of quantification were measured in 190 cases (49.8%). Quantitation data for 13 NPS were also calculated as 10th, 25th, 50th, 75th, 90th, and 95th percentiles (Table 3). In case of a smaller set of samples ($n < 5$), the detected concentration is given. Table 4 presents the quantitative results for the rarely detected NPS.

4 | DISCUSSION

The method was successfully validated according to the guidelines of the Society of Toxicological and Forensic Chemistry (GTFCh) and applied to postmortem hair samples ($n = 1203$) from the period 2008 to 2020. Because of low LODs in picogram range, the method showed very good sensitivity and could be used to detect NPS in postmortem hair samples at trace levels.

Prevalence studies on NPS use are scarce. Anyway, some studies on the prevalence assessed by hair analysis are available.[17,24–28] The NPS prevalence ranges from 0.3% in Hong Kong[27] to 37% in Switzerland.[17] In our study, 381 hair samples tested positive for at least one NPS, resulting in a prevalence of 31.6% among suspected or proven drug users. Because our examined hair samples reflect only about half of the drug deaths due to the two forensic institutions responsible for Berlin, the determined prevalence does not represent all NPS cases from the Berlin region. Nevertheless, the prevalence we found is in the same range as that reported by Larabi et al. (29%) in Paris[24] or Salomone et al. (32.5%) in New York.[26] Prevalence data in all published studies were high compared with conventional drugs of abuse, even though study designs (e.g., kind of studied population

TABLE 4 Postmortem hair concentrations for rarely detected new psychoactive substances (NPS)

NPS	*n*	Hair concentration (ng/mg)
2-Oxo-PCE	1	0.040
3,4-DMMC	2	0.010; 0.011
4-FMC	1	0.042
4-MeO-PCP	1	0.647
5-MeO-DMT	2	0.096; 0.945
Brephedrone (4-BMC)	1	2.73
Butylone	1	0.312
Deschloroketamine	4	0.013–0.044
Ethylcathinone	2	0.012; 0.052
Etizolam	1	0.053
Eutylone	1	0.065
Heliomethylamine	2	0.022; 0.028
Methcathinone	2	0.010; 0.013
MOPPP	1	0.019
NEB	2	0.032; 0.158
Pentedrone	2	0.015; 0.061
Pentylone	1	0.024
α-PPP	2	0.011; 0.012
Pyrazolam	1	0.056
Pyrovalerone	1	0.020
U-47700	2	0.010; 0.333

and compounds, age, sex, hair length) are different.[24] Furthermore, due to the different sociodemographic characteristics, they are difficult to compare. Moreover, they do not allow the real evaluation of prevalence in general population.[28]

Most of the NPS users presented in our study were male and in their mid-thirties. The age and sex distribution are comparable with literature.[29–31] Our study further shows that 3.6% of positive cases were under 20 years of age, indicating the use of NPS in a young population. This is in line with data from the 2019 European School Survey Project on Alcohol and Other Drugs use (ESPAD), which reported a lifetime prevalence for NPS of 3.8% among 15- to 16-year-old students in Germany.[32]

Based on epidemiologic data from six federal states (Bavaria, Hamburg, Hesse, North Rhine-Westphalia, Saxony, and Thuringia), Gomes de Matos et al.[33] provided the first report of regional patterns in NPS and methamphetamine consumption in Germany. The lifetime prevalence of NPS use among persons aged 18 to 64 varied between 2.2% in Bavaria and 3.9% in Hamburg. However, multivariate analysis revealed no statistically significant differences between the states.[33] In a questionnaire-based study of substance use and prevention programs in Berlin's party scene, Betzler et al.[34] reported a slightly higher lifetime prevalence of 15.9% for synthetic cathinone use. In contrast, we found a higher prevalence. The difference is most likely explained by the selection of our cases of death (with a drug history), or by an unknown consumption of NPS because these compounds can be adulterants and replacements in traditional illegal drugs.[35,36] Consequently, substance use is usually underestimated when comparing self-reports in questionnaire surveys with measures of biological markers.[37]

Changes in prevalence were monitored over a time span covering a period before its banning in Germany as well as a few years after banning. No clear trend was visible. Only some substances showed a trend toward an increase or decrease. However, this should not be overinterpreted because many substances were only detected in a few cases ($n < 5$). Some NPS are partially replaced in a few months, indicating their short lifespan.[38] This could be a reason why some analytes (e.g., 2C-B-FLY, 4-MEC, amfepramone, Bromo-DragonFLY, brotizolam, buphedrone, delorazepam, dibutylone, diclazepam, ethylone, MDPPP, methedrone, methylbuphedrone, MPBP, MPPP, naphyrone, α-PBP and p-fluorofentanyl) were not detected in our study. On the other hand, it is possible that other compounds were used. Further, only a few 2C-X compounds and new opioids were found, possibly because of the delayed spread of these compounds or more likely because these substances are highly potent. Due to this, there are active at very low doses, which reduce the detectable levels in hair, especially in case of single or sporadic exposure.[25] This could also be one reason for the large number of qualitative findings in our study.

In the present study, *N*-ethylamphetamine was the most frequently detected substance (range: 0.0101–0.633 ng/mg). It is a Schedule I drug and can be used as a recreational prodrug, while its prevalence and potency are less than amphetamine's.[39,40] *N*-ethylamphetamine was first reported to the EU early warning system in 2014.[41] Subsequently, we observed an increase in positive cases, although we already detected positive cases in previous years. Unfortunately, no published hair concentrations were found for comparison for *N*-ethylamphetamine.

On the contrary, Romanek et al. described the demographics of a large single-center series of cathinone users in Southern Germany for the years 2010 to 2016.[30] They observed a shift from methylone use in the earlier years toward MDPV and 3-MMC in more recent years. Elliott et al. found in a 3-year review of NPSs in casework (2010–2012) in the UK mostly mephedrone and 4-MEC.[42] In our study collective, we were able to detect mephedrone and α-PVP second most frequently after *N*-ethylamphetamine, and methylone in only a few cases. We did not detect 4-MEC in any case. These different findings may reflect local availability of NPS. Notably, the use of synthetic cathinones is constantly shifting.[26]

Quantitation of NPS in postmortem hair samples showed a wide range of concentrations. For example, 4-FA was detected with the largest concentration range, with a maximum concentration of 113 ng/mg. On the contrary, the measured concentrations for most of the remaining NPS were interestingly in the lower nanogram range, suggesting either occasional exposure to these substances, or low rate of incorporation into the keratin matrix.[28] Larabi et al. described similar concentration ranges in hair and suggested that two populations can be distinguished based on the low median of hair concentrations. Hair concentrations of occasional NPS users would be in the pg/mg range and of regular users in the ng/mg range.[24]

In general, our measured hair concentrations are similar to literature data.[24–26,43–46] Hair concentrations of some analytes showed a large variation. For better comparability and interpretation of positive cases in routine work, some measured NPS concentrations were presented as percentiles. It has already been shown that the percentile plot of concentrations is suitable for this purpose, even though the statistical power is low for some analytes due to the still small number of positive cases. The lower range (minimum to 25th percentile) is assumed to be associated with rare to moderate drug use, the middle range (25th to 75th percentile) with occasional use, and the upper range (≥75th percentile) with heavy use.[18,47] Recently, Musshoff et al. presented the concentration distribution of more than 100 drugs and their metabolites in forensic hair samples.[47] In this comprehensive study, hair concentrations of two synthetic cathinones (mephedrone and MDPV) were also presented in percentiles. In comparison, our calculated percentiles for these cathinones are slightly lower, which probably can be attributed to differences in hair extraction protocols (e.g., ultrasonic bath at 50°C for 5 h in 3 ml methanol) as well as different drug behavior of users.

Some cathinones are also used therapeutically. For example, bupropion is used for treatment of major depressive disorders.[48] Ramírez Fernández et al. reported bupropion concentrations between 0.05 and 0.6 ng/mg in hair of patients from workplace drug testing with medication history.[49] Methling et al. investigated postmortem hair samples from cases with positive results for antidepressants and antipsychotics in blood, urine or organ tissue and presented bupropion concentrations in percentiles (mean: 0.0701 ng/mg; median: 0.0164 ng/mg).[50] The detected concentrations for bupropion (0.0119–3.47 ng/mg) in our study are much higher and probably indicate abuse of this substance.

However, positive hair results from postmortem cases should be interpreted with caution, as false-positive results may occur due to external contamination (e.g., caused by smoke, dusts, individual body, or putrefactive fluids).[18,51] This issue should also be considered in hair analysis of living individuals. Further, there is no general consensus regarding the hair washing procedure to remove contaminations.[18] Nevertheless, hair analysis can be very useful to assess possible addiction to a substance, especially in postmortem cases, as these cases often do not contain information on the consumption history.

There are some limitations concerning our study. Due to the choice of LC–MS/MS as investigation method, we could not distinguish between mephedrone (4-methylmethcathinone; 4-MMC) and its isomers 2-MMC and 3-MMC. These isomers have the same MRM transitions and retention time. To solve the problem of chromatographic separation, GC–MS analysis may be used.[52] Inclusion of the different metabolites of 3-MMC and 4-MMC to the analysis method or to use a different analytical column for separation would be another possibility to overcome this problem.[53,54] Therefore, the presence of 2-MMC and 3-MMC could not be distinguished from our mephedrone positive cases.

The choice of the study collective can also be discussed. On the one hand, it is a positively biased selection, because the cases have a suspected or proven drug history. On the other hand, we wanted to detect as many NPS positive cases as possible without having to re-examine all old cases. From literature, it is known that poly-drug users often also deal with NPS.[19,55] Interestingly, synthetic cathinones are not only abused by individuals who use stimulants, but also by opioid abusers.[24,30] Furthermore, our investigated target analytes do not represent all potential analytes and subgroups for NPS testing. Some analytes of interest (e.g., synthetic cannabinoids) were not included due to the lack of reference standards.

The stability of the target analytes in the hair matrix is another critical point, which, so far, has not been investigated for such a long period of time. However, hair differs from other human materials (like blood or urine) used for toxicological analysis because of its substantially longer detection window enabling retrospective investigation of past consumption behavior. Because of its solid and durable nature as well as its difficulty to alter, hair analysis can be performed even centuries after growth.[18,56] Another advantage is that hair analysis provides objective data to determine the prevalence, free from possible untruthful reporting of use or biases of unknown intake.[26,35]

To the best of our knowledge, there is no comparative study available until now. This is the largest number of investigated hair samples from postmortem cases and the longest investigated time period reported in our locality and worldwide at the time of writing. Furthermore, this is one of the few studies that presents NPS concentrations in percentiles, which supports an interpretation of NPS concentrations in postmortem hair. For some of the investigated NPS, these percentiles were published for the first time. Quantitative data were also shown for rarely published substances, which broadens the perception widely.

5 | CONCLUSION

In summary, the LC–MS/MS method was successfully updated, validated, and applied to a very large number of postmortem hair samples. It was demonstrated that hair, even after decades of storage, is well suited for prevalence studies. Our findings showed the presence of a wide number of NPS. Over the 13-year period, NPSs were detected in approximately one third of the investigated cases. Many cases were positively tested for more than one NPS in the same time span, demonstrating a poly-consumption behavior.

Overall, the frequency of NPS findings in postmortem hair samples has increased from 2008 to 2020. Our quantitative data can assist other toxicologists in estimating NPS hair concentrations; nonetheless, one's own case circumstances should always be considered. Because some analytes have been detected only rarely, the validity of measured NPS concentrations in hair is limited, and estimation of frequency of abuse should be made with caution. Further research, including a higher number of positive cases, is needed for their reliable assessment.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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