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REVIEW

Novel psychoactive substances (NPS): Update, issues and challenges

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Abstract. Novel psychoactive substances (NPS), which emerged at the beginning of the 2000s have become a serious and internationally complex situation. These emerging drugs are mostly synthetic and analog derivatives of existing controlled drugs. They are sold ubiquitously not only on traditional markets but also through the Deep Web/Dark Web, where their regulation is even harder. The most important groups comprise synthetic cannabimimetics, cathinones and psychodysleptic phenethylamines. The variety and evolution of this kind of substances has given rise to a continuous analytic challenge for their detection and quantification in seizures and biological fluids. Instrumental chromatographic techniques such as HPLC-DAD, GC-MS, LC-MS, UPLC-MS-MS have provided considerable advantages, although there is still a paucity of standards available and lack of awareness of the metabolites that might be generated in vivo. In this review we will provide an overview of each group of substances and their toxicity, as well as of the analytical methods used in seizures and biological matrices.

Key words: *Novel psychoactive substances; NPS; designer substances; synthetic cannabimimetics; cathinones; phenethylamines; recreational benzodiazepines; Opiomimetics.*

The United Nations Office on Drugs and Crime (UNODC) defines *novel psychoactive substances* (NPS) or *emerging drugs* as those substances of ab use, either in a pure form or a preparation, that are not controlled by the 1961 Single Convention on Narcotic Drugs or the 1971 Convention on Psychotropic Substances, but which may pose a public health threat.[1] They are known as "designer drugs", "legal substances" or "legal highs" on the illicit market.

Most of these drugs come from tests performed licitly by researchers of the pharmaceutical industry searching for new "drugs" for the treatment of various illnesses. When a multitude of structures are synthesized (with chemical variations), they are screened thus separating a potentially useful set of substances from those which are useless. Nevertheless, some of the former are on the way to be discarded due to technical problems or possible side and/or harmful effects justifying their exclusion from the most advanced pharmacological research. Their therapeutic value is determined through affinity assays by central nervous system (CNS) receptors or by modification of biochemical reactions in the nerve cell to achieve the efficiency pursued.[2]

We may redefine "designer drugs" (within the context of their abuse) as: *"those non-natural compounds, synthesized in laboratories, of highly diverse structures and initially unregulated, of limited or non-existent therapeutic value, administered to humans to modify the normal functioning of specific and central cell receptors, either by direct action on them or by modification of indirect biochemical reactions leading to their activation, in order to achieve psychostimulation with alteration of the perception of reality, thus causing behavioral changes and unusual moods"* (author's own definition).

In Fig. 1 we can observe the beginning of semisynthetic drugs, from heroin to laboratory-made synthetic drugs (nitazenes, fentanyls, among others) and the new semisynthetic substances, such as benzopyran derivatives HHC, HHP and THC derivatives which have recently appeared. The year 2000 was a milestone for NPS, with the publication of the first reports on cannabimimetics.

Latest reports on the alarming increase of NPS

In 2023, the number of NPS reported to the UNODC increased to 1230, while the number of countries reached 141. This trend is illustrated in Table 1 and Fig. 2. However,

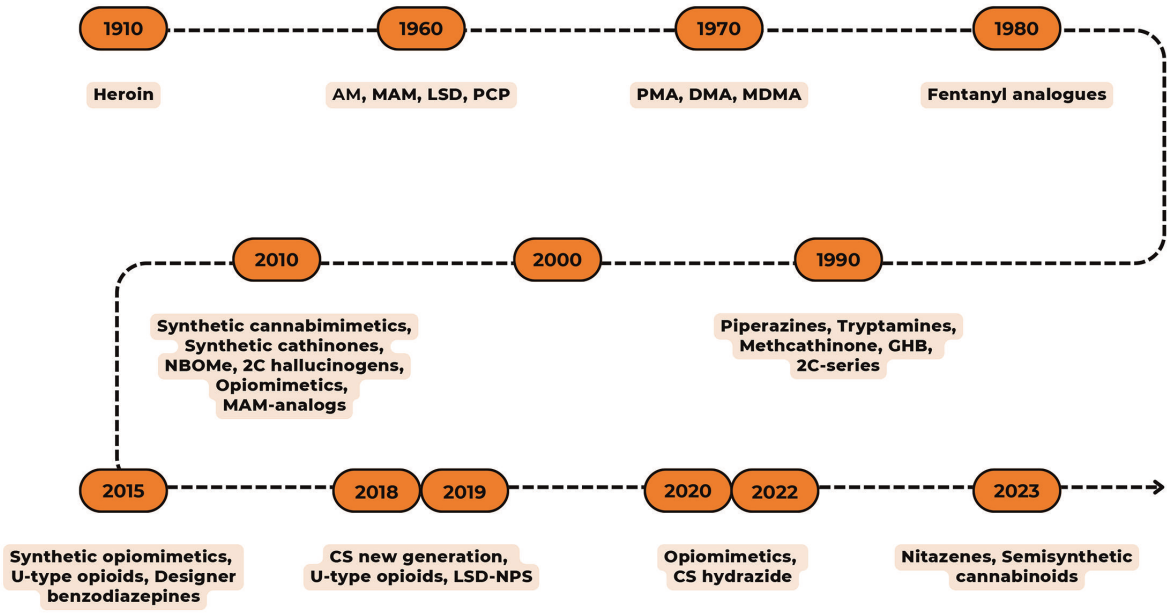

Figure 1. Historical course of the production of semisynthetic and synthetic drugs (NPS and non-NPS).

TABLE 1. Evolution of NPS and number of countries reporting seizures in their territories for the first time.

Year	Number of NPS reported	Total number of countries
2009	166	65
2013	348	90
2014	541	95
2016	644	102
2018	888	118
2022	1,124	134
2023	1,230	141

new structures continue appearing month after month, a clear proof of the globalization of a problem with unforeseeable consequences.[3]

Marketed as "legal drugs" or "designer drugs" and sold openly (including via the internet), they are surpassing the efforts to impose an international control. Criminals hastened to enter this lucrative market. As they are not subject to controls, they are easily introduced in the countries, even by declaring them with names of chemicals customarily used in many industries.[4] Since the toxicity of most of them has not been sufficiently tested, their harm potential is even greater than that of some traditional drugs.

New approaches on the problem of designer drugs

In this section it is worth mentioning the opinion of those who argue that penalization is *per se* a motive for fostering greater drug marketing and consumption. It is paradoxically verified that the use and abuse of these "legal" design drugs, initially non-controlled, continuously and alarmingly increase worldwide.

What is more dramatic is that a wide range of these drugs has been used in sexual assaults or chemical submission. Victims are exposed to substances that cause temporary memory loss and/or distorted sense of time and space, thus being left passively subject to the perpetrator or sex offender, not subsequently retaining any image of the event, as evidenced in the case of GHB (gamma hydroxybutyrate).

"Modus operandi" of design drug dealers

As defined above, an NPS usually appears as a modification of an unregulated substance to prevent authorities from considering it illicit. The spreading of a design drug might be conceived as a cyclical series of events:

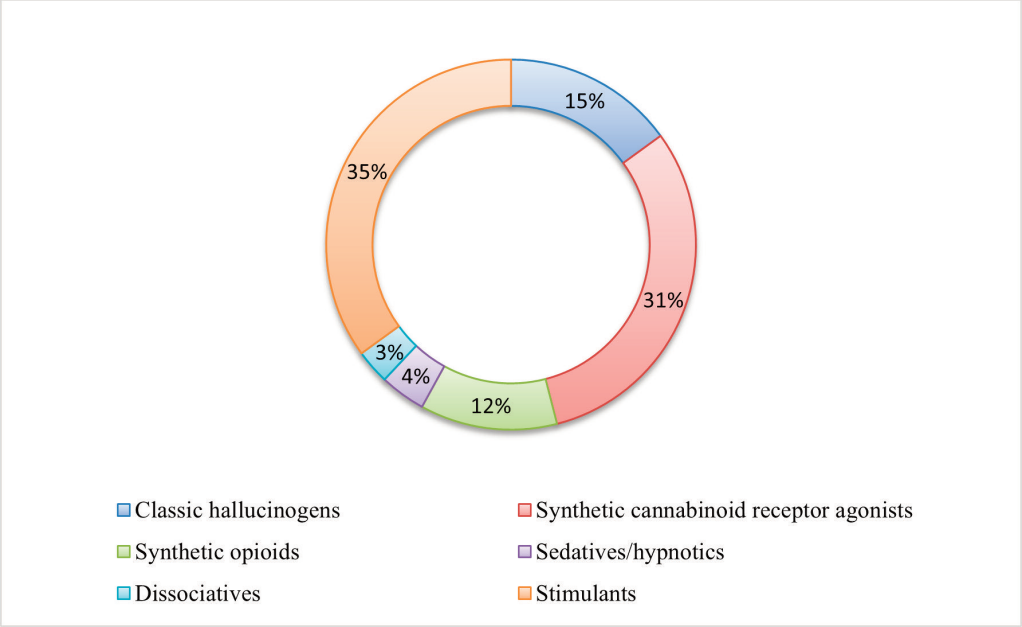

Figure 2. Classification by compound families.

1. Synthesis of a chemical substance which may act similarly to a controlled substance

2. Subsequently the chemical products are marketed as "legal" alternatives to an illicit drug or as "research chemicals, not for human consumption"

3. A small number of users experimenting with the drug report on their experiences via the internet: blogs, forums, videos, among others. If the results are positive, more and more people start using it, thus becoming more popular

4. When laws are updated and this new drug is included on the list of "illegal drugs", the cycle restarts

A new problem arises regarding the control of the sale and distribution of this kind of drugs, since they are chemically modified substances whose structure differs from that of those classified as "illegal substances", thus hindering their identification.[5]

NPS CLASSIFICATION AND DESCRIPTION

Synthetic cannabimimetics (SC)

Overview. A priori the term *cannabimimetic* would seem to be better related to the nature of these compounds, since they do not share the basic, psychoactive structure of cannabis.[2] The term *cannabinoid* would be more in line with those synthetic compounds that have a main chemical nucleus or benzopyran core (e.g. Hu) typical of THC (tetrahydrocannabinol) or CBD (cannabidiol). Most of them are named through combinations of letters and numbers, and the latest as acronyms of structural parts of the molecule (e.g. MB PINACA, where "inaca" corresponds to indazol-carboxamides).[6] Fig. 3 shows the structure of "K2" or JWH-018.[7]

Some compounds appearing on the black market of cannabimimetics do not directly activate the specific cannabinoid receptors CB_1 and CB_2, but they rather inhibit the enzymes that catalyze chemical reactions (e.g. URB447, URB937, which inhibit anandamide degradation) generating toxicity due to the increase in the concentration of endocannabinoid anandamide.

Until the beginning of 2023 SCs amounted to about 381 compounds.[3] Only in Japan, 858 synthetic cannabimimetics have been included on their lists of controlled substances.[8] Most researchers are of the opinion that the international

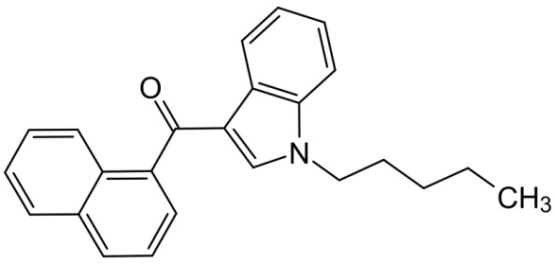

Figure 3. Chemical structure of JWH-018 (K2) (Credits: Mattern R).

situation is critical not only because of the continuous mutation of the active compounds added to the illicit preparations but also due to the scarcity of short and long-term toxicological information. For instance, there is no information regarding the affinity constant for receptor (K_i) for many of these substances. An equally worrying situation are the episodes of acute intoxication they caused among various consumers (even infrequent drug users). Several reports inform about cases of acute psychosis and even acute myocardial infarction among teenagers without previous heart disease.[6,9]

Toxicity. One of the greatest problems of these substances is the lack of information available, particularly regarding their toxicokinetics and toxicodynamics in the human body. Physiological and psychological effects start at minute 10, with a peak at minute 30. Users expound that they experience an effect that is similar or greater to the one presented when smoking cannabis. Nevertheless, many cases of intoxication with severe symptoms have been reported.

In 2011, Dr Colin Kane (pediatric cardiologist in UT Southwestern & Children's Medical Center in Dallas) and his team published an alarming report on three cases of intoxication in 16-year-old teenagers who had used K2 herbal blend.[9] Chest pain, acute myocardial infarction, convulsions, anxiety attacks, increased heart rate and blood pressure, vomiting and disorientation were described. Indazol-carboxamides can also cause hypothermia.

Analytical methods used in seized materials and biological matrices. UNODC's Manual[1,10,11], Namera et al[12] and Worob & Wenthu's reviews[6] feature interesting references. Among them we find the following:

a. Colorimetric assays: the Duquenois–Levine color test, which is widely used in field tests to identify classical cannabinoids such as Δ9-tetrahydrocannabinol, is negative for the synthetic cannabimimetics. The van Urk color test, which is used to identify indole-containing structures, is also negative for these compounds. The use of 2,4-dinitrophenylhydrazine, which reacts with a keto moiety, is capable of reacting with synthetic cannabimimetics, such as the naphthoylindole, phenylacetylindole, benzoylindole, and cyclopropylindole classes, either in powder form or adsorbed onto plant material, and a positive test solution turns from yellow to orange. Nevertheless, the limit of detection has not been stated.[12]

b. Chromatographic and spectrometric methods: mass spectra in GC-MS analysis reflect acceptably the structures of SCs. Naphthoylindole fragmentation pathways have been well studied by gas chromatography-mass spectrometry (GC-MS). Therefore, the identification of these compounds is facilitated by the comparison of the spectra with commercial and open databases. UNODC has published an online open-access manual, with a description of instrumental analytical techniques: gas chromatography-flame ionization detector (GC-FID), GC-MS, liquid-chromatography-mass spectrometry (LC-MS-MS) and a detailed procedure for the identification and quantification of SCs.[10,11] Regarding spectroscopic techniques, such as Fourier-transform infrared spectroscopy (FTIR), they present the analytic difficulty of the herbal base to which SCs are added. Nevertheless, provided there is a previous extraction, i.e. separated from the vegetable matrix, they may be analyzed quite easily. Even so, the spectrum of these extracts differs from that of the one obtained from the pure substance. In general, attenuated total reflectance-Fourier-transform infrared (ATR-FTIR) techniques are suitable for the analysis of isomers in those solids lawfully seized.

Cathinone-related synthetic drugs (psychoactive khat derivatives)

Overview. This is the group of drugs that has had the most continuous use until now. They are synthetic compounds with a chemical structure related to *cathinone,* an alkaloid found in the khat plant (Fig. 4 and 5)[13,14,15], quite similar to amphetamines. According to the report of the National Institute on Drug Abuse-National Institute of Health (NIDA-NIH), the name *"*bath salts*"* given to this kind of substances (due to their macroscopic appearance) may refer to a single substance or a mix of them.

"Bath salts" generally appear as a crystalline white or brown powder (Fig. 6)[16] and are sold in plastic bags or alu-

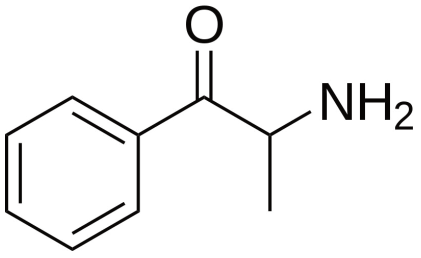

Figure 4. Basic structure of cathinone (Credits: Harbin).

(a) (b)

Figure 5. Carrying "Khat" in a Bangladeshi market (a) and a man dividing the plant into bunches (b) (Credits: Galib E and Frodesiak A respectively).

minum foil packages labelled as "not for human consumption". They are sold on the internet (Deep Web/ Dark Web) and in drug paraphernalia stores under a variety of names such as "Ivory Wave", "Vanilla Sky", "Cloud Nine", among others.

Synthetic cathinones commonly found in the "bath salts" include 3,4-methylenedioxypyrovalerone (MDPV), butylone and eutylone, but there are many others. How these substances affect human brain and their properties, which may vary between one cathinone and the other, is still to be known. They are chemically similar to amphetamines, such as methamphetamine (MA) and ecstasy (MDMA). It is accepted that the methylenedioxy substitution on the aromatic ring gives the drug its entantogenic or empathogenic characteristic. *First-generation* synthetic cathinones derive from substitutions in cathinone structure. However, *second-generation* cathinones have recently appeared, characterized mainly by halogen substitution in the structures of the primary compounds.

Toxicity. "Bath salts" have been marketed as cheap and, not until long, legal substitutes for stimulants, such as amphetamines and cocaine. A study found that MDPV increases dopamine levels in the brain just as cocaine, but it is at least 10 times more powerful.[17] Psychomotor agitation is common since they increase the dopamine (DA) levels in the brain circuits that regulate reward and motion. DA surges in these circuits result in feelings of euphoria and a rise in activity thus increasing heart rate and blood pressure. Incidentally, patients may suffer from dehydration, rhabdomyolysis and renal failure. They are frequently abused and highly addictive, and may lead to death.

Their hallucinatory effects frequently reported are similar to those of the other drugs such as lysergic acid diethylamide (LSD) or MDMA, which increase serotonin levels (5-HT). This can result in serious psychiatric symptoms such as paranoia and panic attacks. Self-harm episodes are not uncommon, as a result of their psychiatric effects and a reduced sensitivity to pain.

The dangers involved in "bath salts" are aggravated by the fact that these products usually contain other ingredients of unknown chemical nature, which may have their own toxic effects. Besides, drug users who believe are buying other substances such as MDMA, may be in danger of receiving synthetic cathinones instead. For example, it has been frequently found that MDMA has been substituted with mephedrone in the tablets sold as ecstasy in the Netherlands.

Analytical methods used in seized materials and biological matrices. Nowadays there are various methods to analyze this kind of substances:

Figure 6. Macroscopic characteristics of mephedrone (Credits: DMTrott).

a. *Colorimetric assays:* the Marquis reagent, which reacts with all nitrogen-containing drugs, is negative for cathinone and mephedrone, but is positive for cathinone analogs that have a methylenedioxy moiety in each molecule, such as MDPV. The cathinone analogs with a methylenedioxy moiety also react with the Chen reagent, which changes to orange in positive tests. However, the limit of detection (LOD) has not been reported. We[18] have modified a method[19] based on the neocuproine reaction with Cu^{+2} salts, thus obtaining an orange color with cathinones, with very few interferences, even of amphetamine derivatives.

b. Instrumental methods: the methods published for the cathinone tests in biological materials generally use a basic extraction (LLE) for cathinones of biological materials. Chromatographic conditions are also simple and do not usually require a special technique. Contributions using LC-MS-MS have been published.[20] Cathinone-detection strategy by LC-MS-MS is almost similar to that of the synthetic cannabinoids; almost all the methods use multiple reaction monitoring (MRM) or selected reaction monitoring (SRM) for sensitive determination. Likewise, by GC-MS or else by time-of-flight-mass spectrometer (ToF-MS) or tandem mass spectrometry (MS/MS) to study the molecular structure. Finally, NMR spectroscopy is used to elucidate molecular structures.

Psychodysleptic phenethylamines

Overview. A little more than ten years ago, a new group of designer substances derived from phenethylamine called NBOMe, "N-bomb" or "Smile" were introduced on the illicit market. They are a powerful family of 5HT2A serotonin receptor agonists, therefore they increase 5-HT activity.

The name "NBOMe" derives from the initials of the chemical groups comprising the structure of this family of drugs: **N**: nitrogen; **B:** benzyl and **OME**: oxymethyl. 2,5-Dimethoxy-N-(N-methoxybenzyl)phenethylamine (NBOMe) was discovered in 2003 and was synthesized as a radioactive tracer for positron emission tomography (PET) in Copenhagen (Denmark). Since it was the first full agonist radioligand for the 5-HT2A receptor, it was promising as a more functional marker of these receptors, which might be involved in mental disorders such as depression and schizophrenia. They are N-(2-methoxy)benzyl derivatives of the "2C compounds" (2,5-dimethoxyphenethylamines with various substituents at C-4), with 33 variations depending on the substitutions occurring on the molecule. Fig 7. shows the chemical structure of 2C-C-NBOMe, a special type of psychodysleptic phenethylamine.[21] In recent years many demethylated derivatives known as "NBOHs" have been detected.

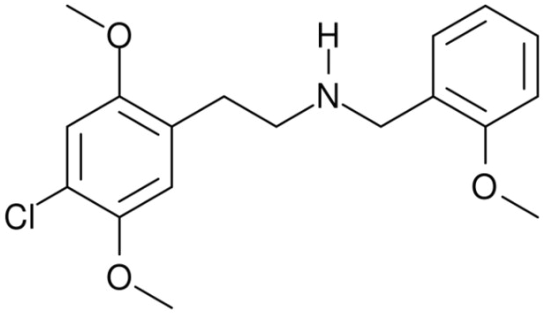

Figure 7. Structure of a special type of psychodysleptic phenethylamine: 2C-C-NBOMe (Credits: C6541).

Formulation and administration. Only active if taken sublingually (also on lips or gums) or intranasally (sniffing). No psychoactivity is present if taken orally. Sold in 0.5-1 mg blotter papers (Fig. 8)[22] which are slightly bigger than those used in the illicit marketing of LSD. They have also been seized as white and crystalline powders.

Toxicity. Among their most relevant effects we should mention sweating, tachycardia, arterial hypertension, psychedelic syndrome, affective lability, distortions in time and sensory perceptions, illusions of movement and impaired judgement. Their clinical effects can be divided into three successive categories from the moment they enter the body[23]:

a. Positive effects ("High"):

- Visual illusions and hallucinations with the open or closed eyes (seeing paths, color changes, fractals, brightness)
- Euphoria, mood boosting, good humor, laughter
- Mental and physical stimulation; associative and creative thoughts; greater awareness and appreciation
- Spiritual experiences: inner peace, introspection, ecstasy
- Sexual thoughts: feelings of love and empathy (entantogenic or empathogenic effect)

b. Neutral effects:

- Generalized change in the state of awareness
- Mydriasis
- Difficulty concentrating
- Unusual body sensations (blushing, piloerection, body energy)
- Changes in time perception
- Slight increase in heart rate
- Yawning

c. Negative effects ("Down", "Comedown" or "Bad trip"). This effect is heightened when the dose is increased. It encompasses:

- Confusion
- Looping
- Nausea
- Insomnia
- Repetitive, recursive and uncontrolled thinking
- Paranoia: fear and panic

Customary doses are: threshold (50-250 μg), low (200-600 μg), median (500-800 μg) and high (700-1500 μg). Taking into account that the doses are in micrograms, measuring the dose-response becomes highly difficult when formulated in powder form. Their effects last between 6 and 10 hours if taken sublingually, or between 4-6 hours if sniffed. The duration of the effects also depends on the dose. The "comedown" takes place between 1 - 4 hours, and the residual effects may last 1 to 7 days. Since the "high" may take 2 hours, the user is subject to the temptation of redosing due to the feeling of lack of effects. Redosing is not recommended, due to the higher risk of toxicity and death. They are highly addictive. It has been proven that in a few months the individual may increase their nightly 1-tablet dose to even 6 to 8, leading to intoxication and even death.

This kind of substance is metabolized mainly by monoamine oxidase enzymes (MAO-A and MAO-B), just like 5-HT. Their long-term effects are still unknown, but it is known that they generate a rapid cross-tolerance with other drugs, such as LSD.

Figure 8. "NBOMe" blotter papers (Credits: Heisenbug).

Analytical methods used in seized materials and biological matrices. Chromatographic methods are the most widely used for this purpose: high-performance liquid chromatography (HPLC) or ultra performance liquid chromatography (UPLC) coupled with several detection systems: MS-MS or time-of-flight mass spectrometry (HRToF-MS). LLE system and solid phase extraction (SPE) are widely use as extraction procedure. Some authors developed techniques for the identification and quantification of 25I-NBOMe and 25C-NBOMe in serum from intoxicated subjects. The samples were collected using SPE technique and 25H NBOMe as internal standard. On the other hand, GC-MS has been successfully used in our country for the identification of 25-I-NBOMe. ATR-FTIR techniques have also been described for the analyses of seized blotter papers.[24]

National and international current legal status. Although their use spread in Europe in 2010, nowadays they are present worldwide. Various cases of deaths have been reported among young people, mainly in Europe, Australia and the United States.[25] In 2013, in Chile, they were seized as blotter papers and since the preliminary tests for banned substances did not detect LSD in their analyses (but a substance that did not appear on the list) the product and the dealers were released. In Argentina, the 33 variations of NBOMe were included in the Presidential Decree No 772 of 2015, therefore nowadays it is a family of substances under control. Initially, seizures coming from Argentine Northwest entered the country as 25-I-NBOMe, a short time after being detected in Chile. Finally, 5 years ago NBOHs started to be identified. They are a group of substances prevailing nowadays in our country as well as in the rest of South America.

Designer benzodiazepines

Overview. Designer benzodiazedpines have increased dramatically in the last two years.[27,28] Although benzodiazepines have always had a long history of abuse, the emergence of pharmacologically potent structures for recreational use may be the beginning of a new surge of these sedatives.[29] The chemical structure of diclazepam, brotizolam and quazepam[30,31,32] can be seen in Fig. 9 and 10, as examples of this kind of substances.

The first designer benzodiazepines available on the internet were: diclazepam, flubromazepam and pyrazolam, none of which have been approved for medicinal use in any country.[33] Since 2020 they have been detected together with other NPS, particularly fentanyl derivatives.

Almost all these compounds have been synthesized as drug candidates by different pharmaceutical companies. Their synthesis, as well as the data from animal testing, are

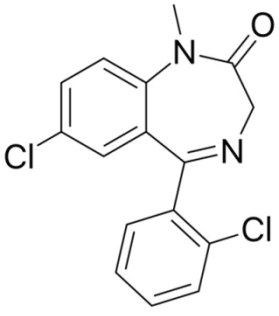

Figure 9. Diclazepam as an example of designer benzodiazepines (Credits: Vaccinationist).

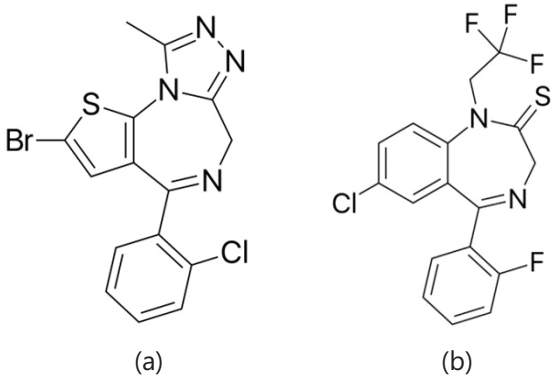

Figure 10. Designer benzodiazepines: some structures detected in recent months. In order: brotizolam (a) and quazepam (b) (Credits: Calvero).

described in the literature, together with many other potentially viable compounds. Their formulations are marketed as pills, scored tablets or blotter papers in various doses, generally attractively colored. Besides, these drugs are also offered as pure powder at prices as low as US$ 0,05-0,10 per dose.

Toxicity. They are highly active and toxic substances. These design psychoactive substances usually have a long half-life, hence, their remarkable residual effects or hangover, which involve an impairment of the higher cognitive and coordination functions. Their widespread availability through internet suppliers and their low price may facilitate the development of addiction among consumers.

Analytical methods used in seized materials and biological matrices. Immunochemical tests applied in clinical cases and in drug seizures are sensitive enough to detect most designer benzodiazepines. However, some of these substances show a high score of cross-reactions with other congeners, e.g. flubromazolam and diclazepam in serum. MS is necessary for the confirmation, particularly due to the fact that the lack of reference standards would not allow to cover the latest designer benzodiazepines that may continue appearing on the recreational market.

Synthetic opiomimetics

Overview. These substances, featuring a highly varied chemical structure, are opioid receptor agonists. Since the beginning of 2010 they have generated an epidemic in the USA, which has for the first time exceeded the heroin overdose deaths, leading the NIH to declare a national emergency. As reference, of the 68,630 opioid-related deaths registered in 2020, 56,516 were synthetic opioids.[34, 35]

(b)

Toxicity. Some effects of synthetic opioids include relaxation, euphoria, pain relief, sedation, confusion, drowsiness, dizziness, nausea, vomiting, urinary retention, miosis, and respiratory depression. Due to the potency of some of them, death is a possible outcome.[36]

Argentinian massive intoxication. In Argentina, at the beginning of 2022, there was a massive intoxication due to a fentanyl derivative. Through the Ministry of Public Safety of the Province of Buenos Aires samples of the substance used were obtained and subsequently sent for testing, confirming it was a mix of cocaine adulterated with carfentanil.[37] The intoxication caused 24 deaths and more than 100 hospitalized victims.[38,39] This massive event, the first worldwide, allowed us to become aware of the analytic difficulties we are facing due to NPS. In this case, the weight ratio of the cocaine in relation to the agent found after deep tests (carfentanil) was very high, therefore the chances of detecting and quantifying it were not promising. We learned that the interaction between emergency physicians, toxicologists and analytical chemists is essential.

On 2nd February at 6 a.m., six male and one female individuals were admitted in the emergency room of a hospital in the west of the province of Buenos Aires. The clinical assessment showed the following signs: miosis, shock, sensory depression, respiratory distress (bradypnea), psychomotor excitement, seizures, and cardiorespiratory arrest. Some even showed certain rigidity like "wooden" chest, typical of opiomimetics such as fentanyl or its derivatives. As the hours passed, the number of cases increased dramatically. At 2 p.m. the epidemiological situation was: 10 deaths and 59 people in intensive care units. All those admitted to hospital had sniffed "a line of cocaine" bought in the suburbs of the province. The photograph (Fig. 11) shows one of the packages found in the clothes of one of those people hospitalized. The victims were treated with naloxone at high doses and with a continuous infusion drip, in some cases, at higher doses than those used for classical opioids.

Figure 11. Photograph of one of the packages seized.

Subsequent tests allowed to prove the presence of carfentanil in doses of 30-60 micrograms per 200 mg of powder sold as cocaine.[39] The first tests consisted in basic analyses through color reactions, high-performance thin layer chromatography (HPTLC), and ultra-performance liquid

Figure 12. Four powder samples seized, and cocaine standard inserted on the plate. We can see at 254 nm a spot at Rf: 0.64 coincident with the cocaine standard. When we applied eosin Y, we couldn´t see any spot attributable to fentanyl or other derivative as carfentanil.

chromatography method with diode array detection (UPLC-DAD), testing positive for cocaine and negative for fentanyl-like structures. Subsequently and due to the lack of positive indicators for fentanyl derivatives, we proceeded to apply NMR and LC-ToF-MS through MRM analysis. In Fig. 12 we can see the detection of cocaine and related compounds through NMR and absence of piperidine derivatives typical of fentanyl compounds.

The workflow is described in detail below:

1. Preliminary analysis by TLC and UPLC-DAD: initially we decided to apply the basic method by TLC.

- Silica stationary phase (Silicagel GF254)
- Mobile phase: methanol-ammonia (100:1.5)
- Standard: Cocaine - LGC©USA
- Plate development and subsequent sequential revealed:
  a) UV 254 nm
  b) Sprayed with eosin Y (recommended by some authors to reveal fentanyl since opiomimetics are suspected, according to the details of clinical toxicological interventions)

2. Samples analysis by UPLC-DAD:

- Column: Acquity UPLC HSS T3 1.8 µm; 2.1 x 100 mm
- Mobil Phase A: water + 0.1 % formic acid
- Mobil Phase B: Acetonitrile

TABLE 2. Gradient.

Time program (min)	Flow rate (ml/min)	%A	%B
Initial	0,4	90	10
0,5	0,4	90	10
9,0	0,4	10	90
9,1	0,4	90	10
12	0,4	90	10

- Column Temp: 35 °C
- Sample Temperature: 15 °C
- Gradient: see Table 2
- Diode Array Detector: Scan 200 to 400 nm, Spectral and Optical Bandwidth 1.2 nm]

3. Validation of method for cocaine determination: we built the calibration curve with the standard at six concentration and calculated the LOD and LOQ, recovery and robustness of the method. Calibration curve was carried out by plotting peak area vs concentration. The least squares linear regression analysis of the data gave us the equation: $y= 11900x + 11.95$ ($r^2 >0.9997$).

LOD = 3 µg/g; LOQ = 10 µg/g; η = 96.8 %

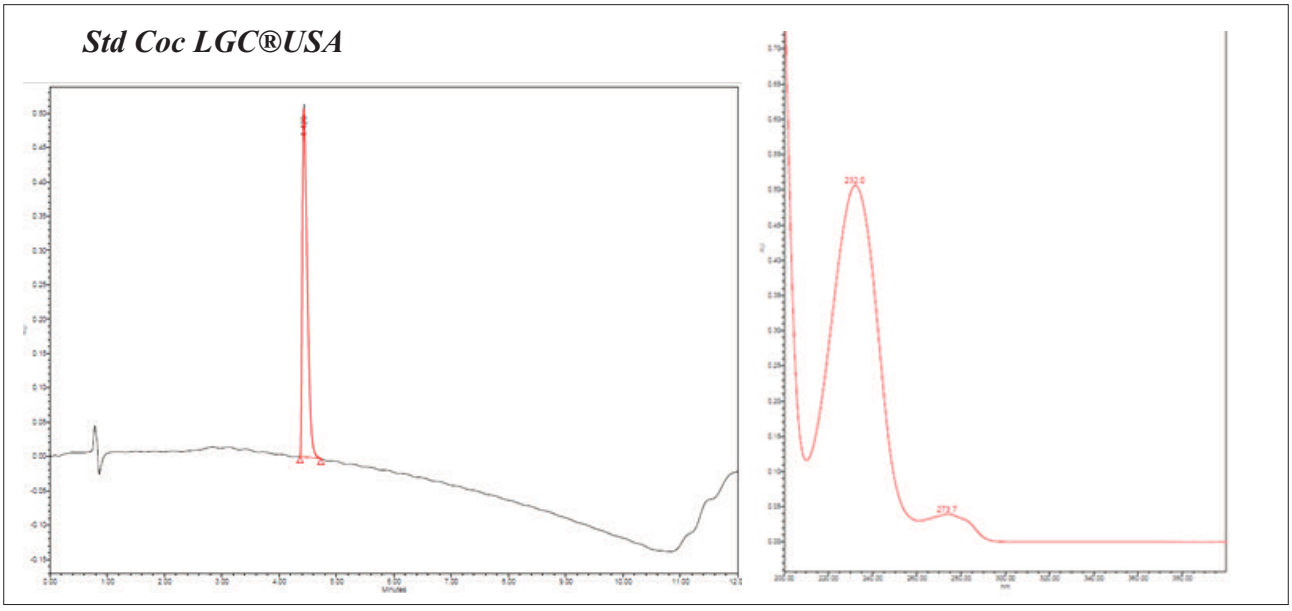

Figure 13. Chromatogram and UV spectra of cocaine standard.

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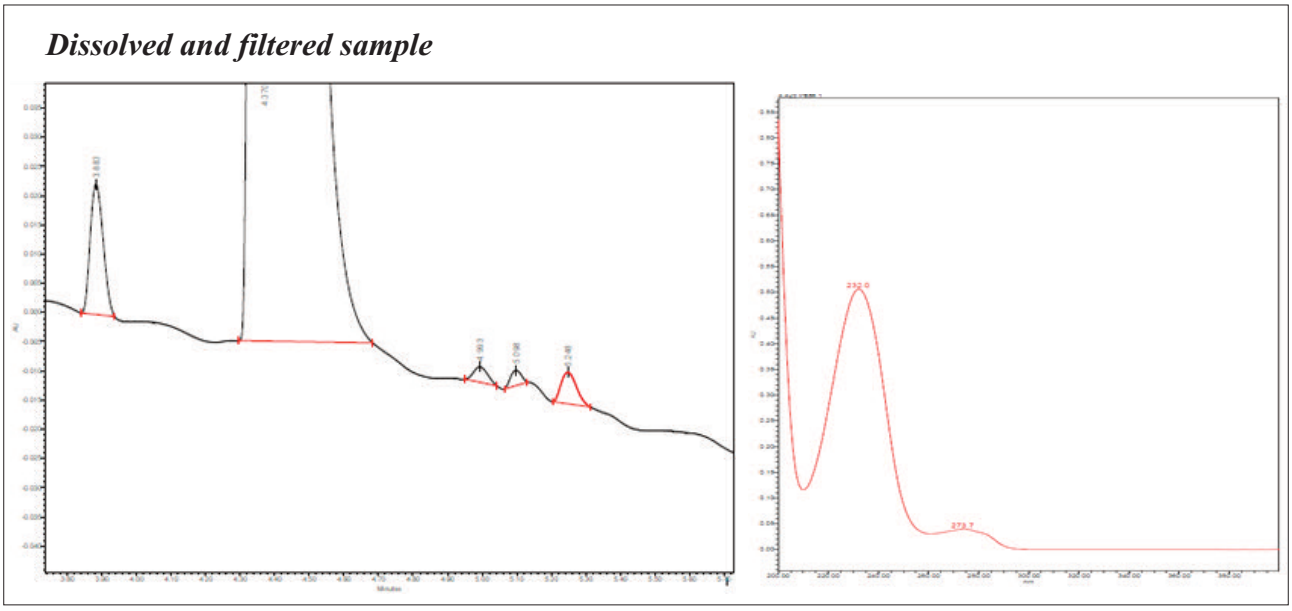

Figure 14. Chromatogram and UV spectra of one powder sample seized.

The conclusions that we were able to draw from what was done in the UHPLC are the following:

a) The samples identified as victim and Lab seized are equivalent, their chromatographic profile is identical
b) In all analyzed samples a high concentration of cocaine and the presence of four unidentifiable chromatographic peaks were observed. The peak retention time 3.88 minutes by its spectrum behaves like a metabolite or substance related to cocaine
c) The quantitative data showed a 51.6% cocaine content with respect to the total weight of the sample

4. Analysis by NMR:

4.1 Sample preparation: the sample was suspended in 1 ml of chloroform-d, shaken, and centrifuged. The solid was subjected to a second wash with chloroform, obtaining the insoluble fraction in chloroform (Fraction I). The chloroform solution was taken to dryness obtaining the soluble fraction (Fraction S).

4.2 Preliminary analysis: it was performed by nuclear magnetic resonance of ^{1}H and ^{13}C to identify major components. Spectra were measured on a Bruker Avance Neo 500 spectrometer (^{1}H 500 MHz, ^{13}C 125 MHz). Directed by Prof. Dr. G. Burton, UMYMFOR-CONICET of the University of Buenos Aires and his team). Fraction S: the spectra were measured in deuterochloroform. NMR spectra showed the characteristic signals of *cocaine hydrochloride* and small amounts of *cis and trans cinnamoyl ecgonine methyl ester hydrochloride*, a natural product that usually accompanies cocaine. Fraction I: spectra were measured in dimethylsulfoxide-d_6 solution. The main component was identified as mannitol. A small remnant of *cocaine and cinnamyl ecgonine methyl ester* is also observed.

5. Analysis by LC ToF-MS (MRM): considering the clinical history, it was decided to use in the first instance a chromatographic method capable of detecting fentanyl and its analogs in the Fraction I, enriched in said components, thus reducing interference. The sample was analyzed in a Bruker MicroToF-Q II mass spectrometer coupled with an Agilent 1200 liquid chromatograph.

Chromatographic conditions:

- Column: Phenomenex Luna 3 µm C18(2) 100 A 100 x 2.0 mm
- Solution A: ammonium formate 5mM adjusted pH 3 with formic cid
- Solution B: 0.1% formic acid in acetonitrile
- Detection: ESI +
- Flow: 0.15 ml/min
- Inyection vol: 20 µl
- Temperature: 25°C
- Running time: 15 min
- Elution program (see Table 3)

Table 3. Elution program.

Time (min)	Disolution A	Disolution B
0,00	87	13
0,50	87	13
10,00	50	50
10,75	5	95
12,25	5	95
12,50	87	13
15,00	87	13

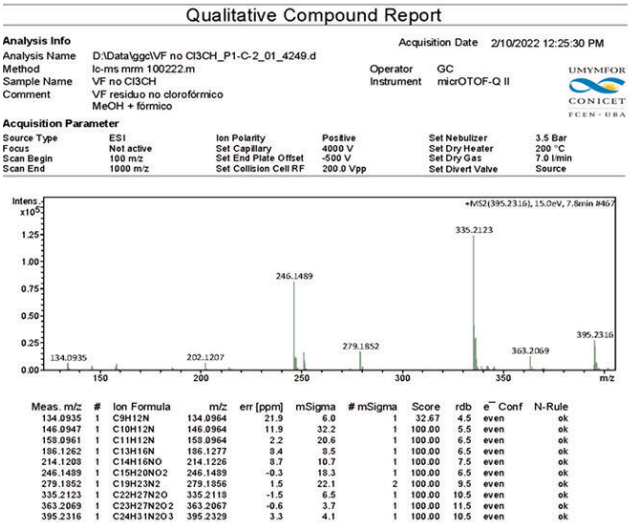

Figure 16. MS spectra (MRM) of signal at Rt 7.8 min.

We obtained the following chromatogram:

It was performed by MRM and MS/MS fragmentation analysis. The carfentanil characteristics fragments are shown in Fig. 16. The structure of each observed ion is presented,

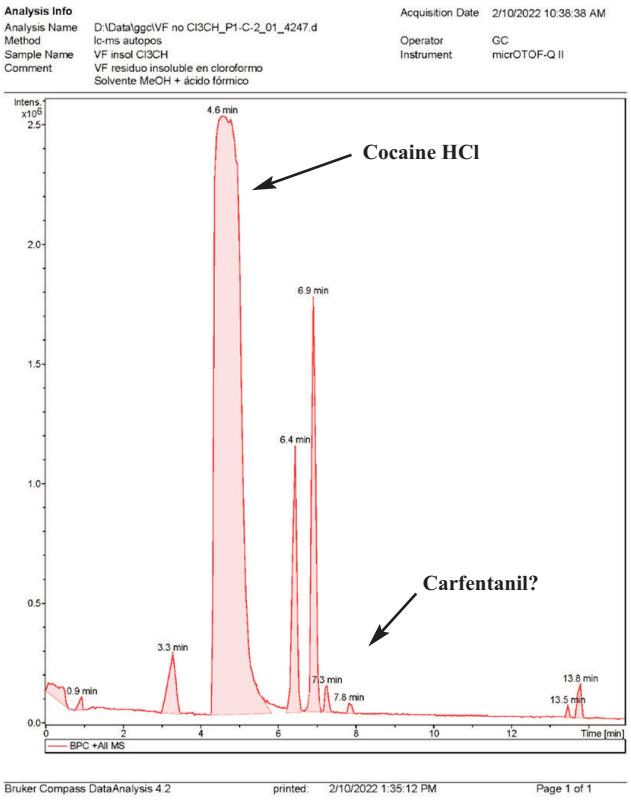

Figure 15. Chromatogram obtained in LC-MS system.

the exact mass measured, and the mass calculated for the formula. In all cases, the isotopic distribution according to the elements present in the formula is observed. In the following spectrum obtained from one of the analyzed samples we can observe the m/z ions obtained as exact mass, compared with the *Waters Corp*. database and the analysis of the ions, with their exact masses. Note the coincidence to the third decimal place of the mass.

6.1 Analysis in biological samples

In the urine of a fatal victim who consumed a line of more than 400 mg, only a signal could be detected by GC-MS that could correspond to carfentanil in non-quantifiable

Table 4. Molecular formulas assigned based on the exact mass value and the isotopic value compared to the calculated values.

RT	m/z	Fórmula (M+H)	Compound
3,3	290,1380	$C_{16}H_{20}NO_4$ (290,1387)	Benzoylecgonine
4,6	304,1538	$C_{17}H_{22}NO_4$ (304,1543)	Cocaine
6,4	330,1644	$C_{19}H_{29}NO_4$ (330,1700)	Cinnamylcocoine (cis/trans)
6,9	330,1693	$C_{19}H_{19}NO_4$ (330,1700)	
7,3	330,1705(+2)	$C_{38}H_{48}N_2O_8$ (330,1700)$[M+2H]^{+2}$	Truxilline
7,8	395,2332	$C_{24}H_{31}N_2O_3$ (395,2329)	**Carfentanil?**

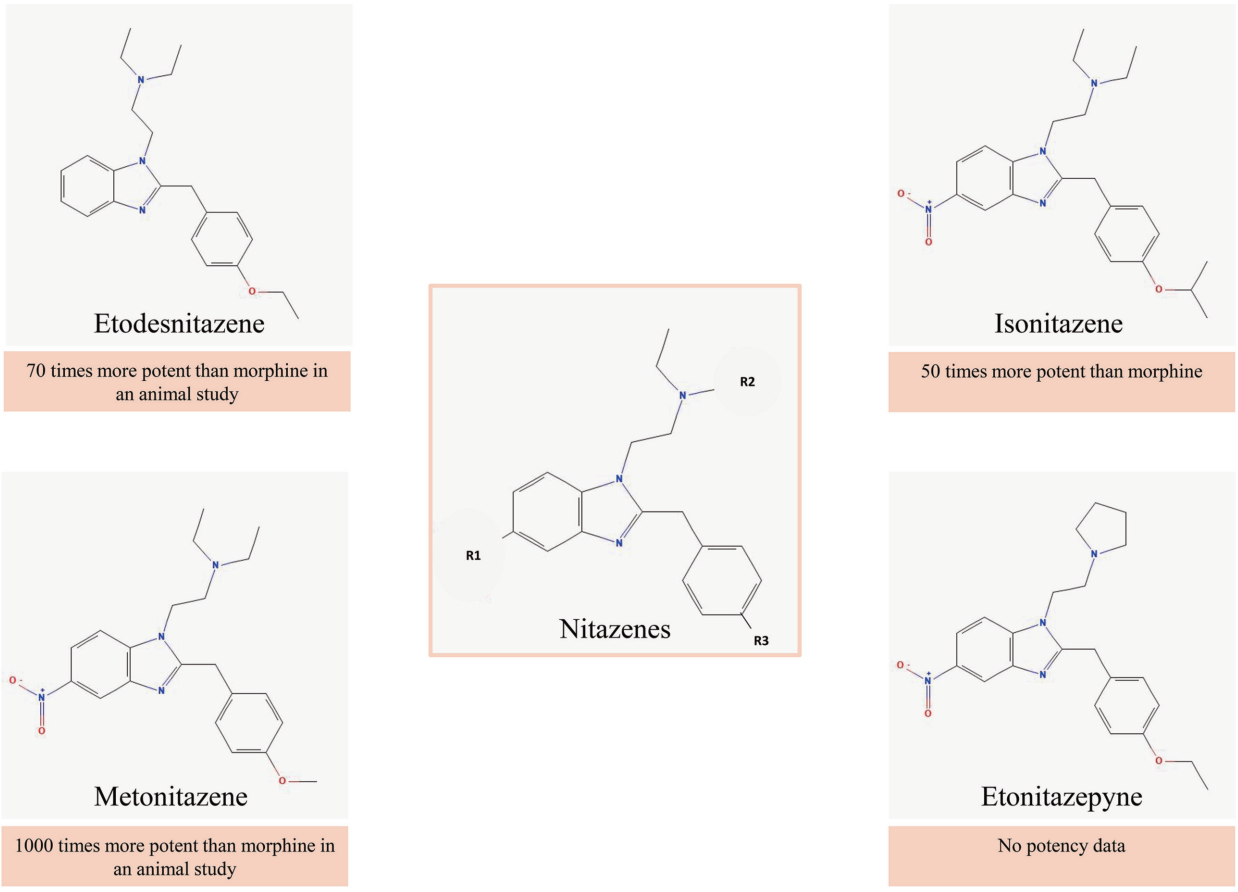

Figure 17. Basic structures of nitazenes and some derivatives that have recently appeared on the illicit market. Please notice that metonitazene is ten times more potent than fentanyl (1000 times higher than morphine) (Source: Pubchem).

values. In other cases, it could not be detected with this methodology, for the reasons already explained in the introduction. It was not possible to analyze the blood and urine of the victims by the methods listed in item 5: LC-ToF-MS/MS.

Nowadays, the twenty fentanyl derivatives are being slowly substituted by a new family of NPS with benzimidazole structure: *nitazenes*.

Nitazenes. Metonitazene and isotonitazene belong to this new kind of synthetic opioids whose structural core is benzimidazole (Fig. 17)[40-44]. They were developed in the 1950s by Ciba-Geigy as painkillers. These agents have been object of recent research as synthetic opioids have become more frequent. However, to date, the data about the prevalence of designer opiomimetics is incomplete, since the traditional post-mortem toxicology tests may not be sensitive to detect these compounds in such low concentrations, particularly when the blood is drawn from a peripheral site instead of central tissues such as the brain, the lungs or the heart.

As with all opioids, respiratory depression is a major risk factor in overdose and even a "wooden" chest may occur. A possible treatment for respiratory depression is the use of naloxone and for muscle rigidity benzodiazepines such as diazepam.[40]

CONCLUSIONS

The varied chemical nature of the different families of NPS constitute an enormous obstacle when it comes to basic and instrumental analysis. The scientific information regarding the level of toxicity of each family and even between the compounds of each group is scarce. Nevertheless, the challenge is not over: new structures, a variety of mixes and assorted adulterants which will make toxicology and analytic clinical assessment even harder are on the horizon.

Declaration of interest
The author declares no conflicts of interest.

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