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COMPREHENSIVE FORENSIC TOXICOLOGICAL ANALYSIS OF DESIGNER DRUGS

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Abstract

Since the introduction of synthetic heroin, designer drugs have been increasing in prevalence in the United States drug market over the past few decades. Recently, "legal highs" in the form of "bath salts" or "research chemicals" labeled "not for human consumption" have become a household term for one such class of designer drugs. While a number of federal and state bans have been enacted, the abuse of these designer drugs still continues while manufacturers have been staying one step ahead of the law with constantly evolving modifications to drug molecular structures. When an intoxication or fatality occurs, presumptive techniques, such as immunoassays, are employed to quickly screen biological specimens for common drugs of abuse. However, since cathinone derivatives are fairly new, few assays have been created for the detection of such compounds. It is hypothesized that during routine drug screens by ELISA or EMIT, the cathinone derivatives and other designer drugs may be missed. In a toxicology lab, a negative screen would not be further investigated and the substances may never be detected. For this reason, it is important to investigate how they may or may not react in presumptive screens, *i.e.,* pre-existing commercial immunoassays.

In this project, 16 different ELISA reagents from Immunalysis, Neogen, OraSure, and Randox were evaluated to determine the cross-reactivity of thirty designer drugs, including 24 phenylethylamines (including MDPV and eight cathinone derivatives), three piperazines, and three tryptamines in serum. In addition, two EMIT reagents were evaluated to determine the cross-reactivity of these same compounds in urine. The study determined the percent cross-reactivity for the compounds in commercial immunoassays targeting amphetamine, methamphetamine and/or MDMA, benzylpiperazine, mephentermine, methylphenidate, ketamine, MDPV, mephedrone, methcathinone, PCP, and cotinine. To further examine cross-reactivity, serum samples spiked in a blind study were analyzed by ELISA and LC-MS to determine false positives or false negatives that may have occurred and to compare the presumptive ELISA results to confirmatory LC-MS analyses.

Cross-reactivity towards most drugs was <4% in assays targeting amphetamine or methamphetamine. Compounds such as MDA, MDMA, ethylamphetamine, and α-methyltryptamine demonstrated cross-reactivities in the range of 30–250%, but data were consistent with both manufacturers' inserts and published literature. Some assays, such as BZP, cotinine, PCP, mephentermine, methylphenidate, ketamine, and MDPV, demonstrated almost no cross-reactivity towards any of the analytes evaluated. When tested against the Randox Mephedrone/Methcathinone kit, cathinone derivatives demonstrated cross-reactivity at concentrations as low as 150 ng/ml. The Mephedrone/Methcathinone kit was not a suitable assay for detecting other more traditional amphetamine-derived compounds but may be more appropriate for screening post-mortem specimens for "bath salts" when putrefactive amines may be present. All other assays demonstrated essentially no cross-reactivity towards any of the analytes evaluated.

In summary, this study determined the cross-reactivity for thirty designer drugs in biological specimens across 18 commercial immunoassay reagents. Very few "false positives" were observed, indicating low cross-reactivity and generally high selectivity of the immunoassays examined. Given these results, a great need exists for additional broad range screening techniques to be applied when analyzing biological specimens for drugs of abuse, specifically the more recent designer drugs.

Table of Contents

Executive Summary

"Designer drugs" are analogs or derivatives of controlled substances that are sold on the street in an attempt to circumvent the legal restrictions placed on scheduled drugs. New designer drugs are constantly emerging onto the illicit drug market and it is often difficult to validate and maintain comprehensive analytical methods for accurate detection of these compounds. Chemical modifications in these substances can be very subtle, leading to virtually unlimited structural variation. As a consequence, there are many hundreds of such entities that have been identified to date. Generally, forensic toxicology labs utilize a screening method, such as immunoassay, for the presumptive identification of drugs of abuse. When a positive result occurs, confirmatory methods, such as gas chromatography (GC) or liquid chromatography (LC) coupled with mass spectrometry (MS), are required for more sensitive and specific qualitative and quantitative analyses. In recent years, the need to study the activities of these compounds in screening assays as well as to develop confirmatory techniques to detect them in biological specimens has been recognized

Designer drugs have been a major topic of concern in Europe for some time and this issue has also become increasingly important in the United States. The United States Drug Enforcement Agency (DEA) has scheduled, emergency scheduled, and even unscheduled a number of these compounds. For example, DEA recently scheduled 26 designer drugs in the cathinone, phenethylamine, and synthetic cannabinoid classes under the Synthetic Drug Abuse Prevention Act of 2012. In addition to the federal legislation, 43 states and Puerto Rico have outlawed synthetic cathinones as of November 2012, according to the National Conference of State Legislatures (NCSL). Many of the states have enacted laws more stringent than those in place at the federal level, with some states banning cathinones as a general class of compounds. Most recently, severe intoxications and fatalities have been reported with new and emerging designer drugs, presenting analytical challenges for detection and identification of such novel compounds.

There is a critical need in the field of forensic toxicological analysis for reliable screening assays for multiple designer drugs in addition to analytical methods optimized for comprehensive screening and confirmation of such drugs in a variety of human specimens for both ante- and post-mortem investigation. The major goal of the present project was to evaluate the performance of commercially available screening immunoassays for detecting a wide range of designer drugs. It is expected that the results of this study will advance the science and practice of forensic toxicology for this important class of drugs.

Clinical and forensic toxicology laboratories utilize screening methods such as immunoassays for a quick, cost-efficient approach to gaining basic information about drug content of a biological specimen such as blood or urine. Immunoassays are designed to indicate the presence (above a certain "cutoff" concentration) of a particular class or type of substance, such as amphetamines. For example, a typical toxicology laboratory may perform a drug screen with individual kits targeting barbiturates, benzodiazepines, cocaine, opiates, and oxycodone. Depending on the nature of the immunoassay (*i.e.*, monoclonal vs. polyclonal), the concentration of the drugs, or the structures of the analogs, some compounds may not be detected. Consequently, while these screens may perform well for known derivatives, they are not ideal for unclassified substances because of the likelihood of unconfirmed positives. In addition, multiple classes of drugs generally cannot be detected and identified in a single test. In regulated workplace drug testing, only a few drugs are targeted and high specificity is a desirable characteristic for antibodies used in that field. However, for post-mortem and human performance drug testing, broad selectivity is desirable.

To date, very few investigations of the cross-reactivity of new designer drugs in standard immunoassays have been reported. Consequently, cross-reactivities still remain unknown for many drugs, particularly the newest compounds such as the cathinone derivatives. Based on the above data, there is a critical need in the field of forensic toxicology for reliable screening assays for multiple designer drugs, in addition to analytical methods optimized for comprehensive screening and confirmation of such drugs in a variety of human specimens for both ante- and post-mortem investigation. Since each manufacturer is likely to employ different antibodies, specificity for individual drugs cannot always be predicted among different types of immunoassays and different sample matrices. Regardless of any cross-reactivity that may occur, it is imperative that the forensic analytical toxicology community be made aware of the results, as screening techniques are limited for designer drugs, particularly the cathinone derivatives.

Two primary hypotheses were tested in this project: 1) Some amphetamine-like designer compounds would not be detected using conventional assays, even when multiple immunoassay platforms were utilized (*i.e.,* false negatives) and 2) Some designer compounds would be detected in assays that theoretically target only amphetamine and methamphetamine (*i.e.,* false positives). In a working toxicology lab, a negative result as in (1) would generally not be further analyzed or confirmed with other methods, with the consequence that the drugs may be overlooked. In contrast, a positive result as in (2) would trigger a confirmatory analysis, although an unknown designer drug would generally not be identified without proper reference standards or a comprehensive chromatographic method. A focus was placed on amphetamine and methamphetamine/MDMA reagents, as one or both of these types of assays are used in routine drug screens by a majority of labs. Since presumptive methods, like ELISA and EMIT, are the first line of screening methods for detecting drugs of abuse, it is necessary to understand how these important drugs can be detected, if at all, by currently available immunoassays, even where cross-reactivity is not expected.

The present project included two primary tasks. Task 1 consisted of determination of the cross-reactivity of designer drugs in common commercially available immunoassays. Task 2 consisted of similar screening evaluation using a series of blind spiked serum samples, with comparison to screening results obtained using a validated LC-QQQ-MS method and high-resolution LC-QTOF-MS analysis. For Task 1, 30 designer drugs from the phenethylamine, cathinone, tryptamine, and piperazine classes were analyzed by 16 ELISA reagents and 2 EMIT reagents in serum and urine, respectively, to determine cross-reactivity. For determination of cross-reactivity by ELISA, samples of drug-free serum (0.5 mL) were fortified with 50 µL of a methanolic spiking solution for analysis. All calibrators, controls, and samples (spiked or authentic) were subjected to a 1:4 (5x) dilution with dilution buffer. The ELISAs were performed using a DSX® Four-Plate Automated ELISA processing System. For analysis by EMIT, samples of drug-free urine (1 mL) were fortified with 100 µL of a methanolic spiking solution for analysis. The urine samples were not diluted prior to analysis, per manufacturer instructions. The EMITs were performed using a V-Twin® analyzer.

When initially assessing cross-reactivity, all analytes were analyzed at a concentration of 10,000 ng/mL in serum or urine. This level was chosen based on the cross-reactivity studies performed by the manufacturers as outlined in the package inserts. If a positive result was obtained, a dose response curve was analyzed to determine the percent cross-reactivity. The concentration of each analyte of interest that produced an absorbance reading closest to that of the positive cut-off control was also calculated. This value is represented by the $A_{sample}/A_{negative}$ with a ratio closest to 1. The positive cut-off level was then divided by the concentration of each analyte with the same absorbance value and expressed as a percent, representing the percent

cross-reactivity.

For Task 2, 22 five mL serum samples were prepared and analyzed in a blind manner (*i.e.,* without analyst knowledge of drug identity or concentration). For this study, drug-free serum was spiked with known amounts of drug reference standards in methanolic solution. Samples were prepared that included single or mixtures of drugs and were prepared at a range of concentrations simulating those expected to be encountered in authentic specimens. The samples were given a unique ID number and frozen at -20°C until analysis by ELISA and LC-MS. For analysis by LC-MS, a solid phase extraction was performed to clean up the sample and isolate the drug. Serum samples (1 mL) were diluted with 2 mL of phosphate buffer and extracted before analysis by LC-QQQ and LC-QTOF. Qualitative screening by LC-QQQ-MS employed an Agilent 1290 Infinity Binary Pump LC coupled to an Agilent 6460 triple quadrupole MS/MS with Jet Streaming technology and electrospray ionization (ESI) using Agilent MassHunter software. Samples were also analyzed by high-resolution MS for confirmation and library matching. For this purpose, an Agilent 1290 Infinity Binary Pump LC coupled to an Agilent 6530 quadrupole time-of-flight (QTOF) was utilized. Data acquisition was performed in full-scan mode with positive ESI.

Upon analysis by the sixteen ELISA reagents in Task 1, several kits did not exhibit cross-reactivity with any of the analytes of interest: Neogen Ketamine, Neogen Methylphenidate, OraSure PCP, and OraSure Cotinine. This was not unexpected, due to the structural differences between the analytes targeted by the kit and those under investigation here. Upon investigation of the amphetamine-targeting kits, it became apparent that these reagents are quite selective. After examining the analytes of interest by methamphetamine-based reagents, it was evident that the results were comparable to those using kits targeting amphetamine, except with regard to the cathinone derivatives. The Immunalysis Methamphetamine, Neogen Methamphetamine/MDMA, and OraSure Methamphetamine kits displayed positive test results for MDEA, MDMA, and ethylamphetamine at low concentrations, with cross-reactivities between 15 and 250%. While cross-reactivity was less than 2% for the cathinone derivatives using the Immunalysis or Neogen methamphetamine reagents, with positive test results at levels as low as 1,250 ng/mL, the OraSure assay demonstrated greater cross-reactivity for this class of compounds. Positive test results for mephedrone, methcathinone, methylone, 4-MEC, flephedrone, butylone, and methedrone were still observed at concentrations as low as 40-450 ng/mL, with cross-reactivity values in the range of 2-25%.

While these findings indicate that the OraSure methamphetamine reagents are less specific than those from Immunalysis or Neogen, they also demonstrate that this assay kit may be a viable screening tool for presumptively detecting “bath salts” in biological fluids at concentrations that can be encountered in forensic specimens, without necessarily targeting overdose levels. It is important to note that the OraSure Methamphetamine kit is designed for screening in oral fluid, so it may not be commonly used by laboratories screening other matrices and may require additional validation. The Randox MDPV kit was extremely selective, with only butylone demonstrating cross-reactivity at levels as low as 150 ng/mL. The Randox Mephedrone/Methcathinone kit was less specific, since the other cathinone derivatives were still positive at 150 ng/mL when compared to the positive cut-off control.

The values for cross-reactivity for the compounds of interest by EMIT were generally consistent with literature and the package inserts. The Amphetamines kit uses *d*-methamphetamine as the cut-off control, so the results for amphetamine were to be expected. The cross-reactivities with MDA, MDEA, and MDMA were comparable to those listed in the kit insert. From the behavior of AMT in the serum ELISAs, the reactivity of AMT was not unexpected, though it was not very high. With regard to the Ecstasy kit, the cross-reactivities for

MDA and MDEA were comparable to those stated in the package insert. Surprisingly, butylone exhibited some cross-reactivity down to 4,000 ng/mL, which may indicate where antibody binding occurs. However, the cathinone derivatives remained undetected by these reagents at high concentrations and would not be expected to be identified in the urine by either of these reagents.

After cross-reactivity was determined for these compounds, twenty-two serum samples were spiked in a blind study and screened by all 16 ELISA reagents, followed by analysis by LC-MS in order to compare the techniques. The results obtained by LC-MS confirmed many of the "false-positives" obtained during the ELISA screening of these samples. For example, the reagents targeting methamphetamine gave positives results for samples that did not contain methamphetamine and the reagents targeting amphetamine gave positives for samples that did not contain amphetamine. The MDPV kit successfully detected the analyte in one spiked sample but gave indeterminate results for several samples that did not contain this compound. The Mephedrone/Methcathinone reagent was able to successfully detect cathinone derivatives in 10 out of the 11 samples which contained such analytes. The single nondetect sample was determined to contain flephedrone at a very low concentration that was also undetected by the immunoassays. Four samples that would have been assumed negative by immunoassay were determined to contain 2C compounds when analyzed by LC-MS. The blind spiked samples were also analyzed by LC-QTOF-MS to confirm the LC-QQQ-MS results using high-resolution mass spectral library matching. The samples were analyzed in full-scan mode and compared to a full-scan library, while also considering retention time in identification of the analyte. With the exception of drugs that were not included in the mass spectral library, all of the compounds were successfully identified with high confidence by high-resolution parent mass data.

In summary, in this comprehensive study thirty designer drug entities from the phenethylamine, tryptamine, and piperazine structural classes were evaluated against sixteen different commercial ELISA reagents and two commercial EMIT reagents in order to determine cross-reactivity. Since few assays are currently available that target these analytes, particularly the "bath salts", it was important to understand how such drugs may react, especially in presumptive screens. The first hypothesis proposed in the present investigation, i.e., that some novel drugs would not be detected in conventional screening immunoassays, was confirmed. For example, cathinone derivatives and other designer drugs outside the realm of the traditional phenethylamines, such as amphetamine, methamphetamine, and MDMA were not routinely detected. This observation demonstrates that forensic toxicological screening approaches will not be able to solely rely on immunoassays, at least those currently available in the commercial marketplace.

The second hypothesis, *i.e.,* that some designer compounds would be detected in assays that target only amphetamine and methamphetamine, was also confirmed, although cross-reactivity with untargeted drugs was generally limited. MDA, MDMA, ethylamphetamine, and AMT demonstrated cross-reactivity at low concentrations, but results were consistent with those published by the manufacturer or as reported in the literature. Cross-reactivity towards the cathinone derivatives was also found to be limited. However, the cathinone derivatives did demonstrate cross-reactivity at low concentrations (<150 ng/mL) when analyzed against the Randox Mephedrone/Methcathinone kit. While this reagent seemed less selective, there was no cross-reactivity with other amphetamine-like compounds. This finding suggests that the Randox kit may be useful for detecting a wide range of "bath salts" in post-mortem specimens, without the usual interference from putrefactive amines formed during decomposition. Overall, a

majority of the kits analyzed, particularly those targeting phenethylamines, did not exhibit cross-reactivity with the compounds of interest, particularly the cathinone derivatives.

An important conclusion from these data is that current immunoassay-based screening methods may not be ideal for presumptively identifying most designer drugs, including the "bath salts". Laboratories should be aware of the issue of cross-reactivity (or the lack thereof) when performing routine screens so that these types of compounds are not overlooked. While oral fluid may not be a commonly analyzed matrix, the results obtained for the cross-reactivity of the cathinone derivatives in serum by an oral fluid specific reagent (i.e., the OraSure Methamphetamine kit) suggests its possible use for detecting such compounds. However, this would require extensive validation for this matrix and would not necessarily be applicable to every cathinone derivative.

Recently, there has been a trend toward the introduction of new immunoassays with specificity for individual designer drugs or groups of drugs, a development that can, at least to some extent, help address this problem. Alternatively, as more laboratories move towards LC-MS/MS as an in-house analytical tool, screening methods for such analytes will likely gravitate towards higher specificity approaches, in particular high-resolution, high mass accuracy MS. More advanced analytical techniques, such as LC-MS, are required for the identification of these compounds, as demonstrated by the LC-QQQ and LC-QTOF analyses. Toxicology labs should and will continue to move towards LC-MS or other advanced techniques for the detection of these compounds in routine screenings of biological specimens.

There are some limitations to the data produced in this study with regard to generalization to the forensic toxicological screening process. For example, the study only examined 30 drugs, of which only eight were cathinone derivatives. In addition, no synthetic cannabinoids were included in the investigation. Many more compounds exist in the "designer drug" universe, and novel entities continue to be synthesized. Consequently, the current research cannot be considered comprehensive or complete with regard to all potential designer drug entities. Another limitation is that there are several commercially available screening platforms that were not evaluated in the study, and new assays with enhanced designer drug specificity continue to be introduced. These also need to be systematically evaluated with respect to drug cross-reactivity. One problem that was encountered in the present study, which necessitated a change in protocol, was the lack of availability of authentic specimens for comparative screening by immunoassay and LC-MS based methods. However, it is believed that the use of blind spiked specimens as an alternative approach was successful in achieving the original goals of Task 2 of the project.

As noted above, future cross-reactivity studies should include evaluation of additional individual designer drugs, drug classes, and commercial immunoassays. As the designer drugs in the market become more diversified, additional analytes may need to be examined for cross-reactivity. While computational modeling can help in predicting cross-reactivity [43], actual screening experiments in working laboratories are necessary to confirm these predictions. In addition, future research should include similar cross-reactivity studies with large numbers of authentic forensic specimens, in order to provide insight into the effects of individual variability in sample matrix characteristics and possible drug-drug interactions that could influence assay cross-reactivity.

Despite the utility and widespread use of immunoassay-based screening in forensic toxicology, an important conclusion from these studies is that current screening methods may not be ideal for presumptively identifying the universe of designer drugs. The trend toward the introduction of new immunoassays with specificity for individual designer drugs or groups of drugs is a development that can, at least to some extent, help alleviate this problem. However, it

is unreasonable to expect, in view of the time and effort necessary to produce them, that assay probes specific for each of the hundreds of individual designer drug entities will be available any time soon. Alternatively, as more laboratories move towards LC-MS/MS as an in-house analytical tool, screening methods for such analytes will likely gravitate towards higher specificity approaches, in particular high-resolution, high-mass-accuracy MS. However, despite recent advances in designer drug analysis by mass spectrometry, currently available MS-based screening methods are generally limited to analysis of up to several dozen relevant drugs, often from only one of the two primary designer drug classes (i.e., amphetamine-like stimulants and synthetic cannabinoids). Consequently, there is a great need for research to validated new MS-based methods for screening and confirmation of the many hundreds of designer drugs potentially present in forensic toxicological specimens, in addition to methods capable of detecting and identifying novel compounds.

I. Introduction

1. Statement of the problem: "Designer drugs" are analogs or derivatives of controlled substances that are sold on the street in an attempt to circumvent the legal restrictions placed on scheduled drugs. New designer drugs are constantly emerging onto the illicit drug market and it is often difficult to validate and maintain comprehensive analytical methods for accurate detection of these compounds. Chemical modifications in these substances can be very subtle, leading to virtually unlimited structural variation. As a consequence, there are many hundreds of such entities that have been identified to date. Generally, forensic toxicology labs utilize a screening method, such as immunoassay, for the presumptive identification of drugs of abuse. When a positive result occurs, confirmatory methods, such as gas chromatography (GC) or liquid chromatography (LC) coupled with mass spectrometry (MS), are required for more sensitive and specific qualitative and quantitative analyses. In recent years, the need to study the activities of these compounds in screening assays as well as to develop confirmatory techniques to detect them in biological specimens has been recognized [1].

Designer drugs have been a major topic of concern in Europe for some time and this issue has also become increasingly important in the United States. The United States Drug Enforcement Agency (DEA) has scheduled, emergency scheduled, and even unscheduled a number of these compounds. For example, DEA recently scheduled 26 designer drugs in the cathinone, phenethylamine, and synthetic cannabinoid classes under the Synthetic Drug Abuse Prevention Act of 2012. In addition to the federal legislation, 43 states and Puerto Rico have outlawed synthetic cathinones as of November 2012, according to the National Conference of State Legislatures (NCSL). Many of the states have enacted laws more stringent than those in place at the federal level, with some states banning cathinones as a general class of compounds. Most recently, severe intoxications and fatalities have been reported with new and emerging designer drugs, presenting analytical challenges for detection and identification of such novel compounds [1-19].

There is a critical need in the field of forensic toxicological analysis for reliable screening assays for multiple designer drugs in addition to analytical methods optimized for comprehensive screening and confirmation of such drugs in a variety of human specimens for both ante- and post-mortem investigation. The major goal of the present project was to evaluate the performance of commercially available screening immunoassays for detecting a wide range of designer drugs. It is expected that the results of this study will advance the science and practice of forensic toxicology for this important class of drugs.

2. Literature citations and review: Clinical and forensic toxicology laboratories utilize screening methods such as immunoassays for a quick, cost-efficient approach to gaining basic information about drug content of a biological specimen such as blood or urine for both ante- and post-mortem analysis. Immunoassays are designed to indicate the presence (above a certain "cutoff" concentration) of a particular class or type of substance, such as amphetamines. For example, a typical toxicology laboratory may perform a drug screen with individual kits targeting barbiturates, benzodiazepines, cocaine, opiates, and oxycodone. Depending on the nature of the immunoassay and the detection probes utilized (*i.e.,* monoclonal *vs.* polyclonal antibodies), the concentration of the drugs, or the structures of the analogs, some compounds present in the specimen may not be detected. Consequently, while these screens may perform well for known derivatives, they are not ideal for unclassified substances because of the likelihood of false negatives and/or unconfirmed positives. In addition, multiple classes of drugs generally cannot be detected and identified in a single test [20, 21]. In regulated workplace drug

testing, only a few drugs are targeted and high specificity is a desirable characteristic for antibodies used in that field. However, for post-mortem and human performance drug testing, broad selectivity is desirable.

The structures of many of the stimulant-type designer drugs closely resemble those of amphetamine, methamphetamine, and MDMA (See Table 1 for drug abbreviations). Based on the presence of common structural groups, it is not unreasonable to expect that some of these drugs may also be recognized by the antibodies used in the commercial immunoassay kits [22]. However, antibody binding affinity (and thus cross-reactivity) is not based on structural similarity alone, so it is difficult to predict the activity since antibody and conjugate designs are typically proprietary information. Specific immunoassays are not yet widely available for new designer drugs of this class, although some limited research has been performed to characterize the performance of pre-existing immunoassays for such compounds (see below). One commercial provider (Randox Laboratories) recently released two assay kits for the presumptive identification of "bath salts", one targeting MDPV and another targeting mephedrone/methcathinone. To date, very few investigations of the cross reactivity of new designer drugs in standard immunoassays have been reported. Consequently, cross-reactivities still remain unknown for most drugs, particularly the newest compounds such as the cathinone derivatives.

There have been several studies that have reported cross-reactivity values for a number of compounds in a variety of assays using spiked specimens. For example, Park *et al.* described the cross-reactivity of several amphetamine analogs in human urine using Abbott TDx (amphetamine cutoff 300 ng/mL), Vitalab Selectra (methamphetamine cutoff 1000 ng/mL), Accusign MET (on-site test kit, methamphetamine cutoff 1000 ng/mL), and SD Bioline MET (on-site test kit, methamphetamine cutoff 1000 ng/mL) [23]. They demonstrated high cross-reactivities for MDA, MDMA, and MDEA for all of the kits, with confirmation by GC-MS. Crooks *et al.* investigated an alternative matrix, oral fluid, in order to assess screening assays for amphetamines and methamphetamines [24]. Roche DAT assays (amphetamines cutoff 40 ng/mL; methamphetamines cutoff 40 ng/mL) were evaluated with MDA, MDMA, MDEA, MBDB (N-methyl-1,3-benzodioxolylbutanamine), PMA (*para*-methoxyamphetamine), and BDB (3,4-methylenedioxy-α-ethylphenethylamine). Cross-reactivity was reported for PMA, MDA, and BDB for the amphetamine kit while MDMA, MDEA, and MBDB showed significant reactivity using the methamphetamine kit.

Cody *et al.* used fluorescence polarization immunoassays to detect a group of designer drug analogs and metabolites in urine with two Abbott TDx kits (Amphetamine class; Amphetamine/Methamphetamine II) [25]. High cross-reactivity was reported for MDA, MDMA, MDEA, and 4-hydroxymethamphetamine for both kits but many compounds still were either undetected or detected as positives with only one set of reagents, indicating that a negative immunoassay result does not mean an amphetamine analog is not present. Apollonio *et al.* also completed a study examining the cross-reactivities of amphetamine-type drugs using two BioQuant Direct ELISA (enzyme-linked immunosorbent assay) kits (Amphetamine; Methamphetamine) [26]. Using a PBS (phosphate buffered saline) matrix, high cross-reactivity at 50 ng/mL was reported for MDA, PMA, 4-methylthioamphetamine, and phentermine with the amphetamine kit. They concluded that the kits are useful for the examination of blood, urine, and saliva at drug concentrations at 6 ng/mL without interferences from putrefactive amines. Recently, Kerrigan *et al.* published a more comprehensive evaluation of psychedelic phenethylamines (*i.e.,* 2C-B, 2C-I, DOB, DOI) [27], while cross-reactivities for additional phenethylamines were reported by Nakanishi et al. [28]. A large-scale study by Nieddu *et al.*, focused on cross-reactivity of over forty amphetamine-type drugs in urine using EMIT

immunoassays. Their focus included compounds in the 2C, DO, and ALEPH series and reported minimal cross-reactivity with the Amphetamine and Ecstasy assays. High cross-reactivity was reported for MDIP (3,4-methylenedioxy-*N*-isopropylamphetamine), MDBZ (3,4-methylenedioxy-*N*-benzylamphetamine), and MDCPM (3,4-methylenedioxy-*N*-cyclopropylmethylamphetamine) [29]. However, these studies have incorporated few designer drug compounds or synthetic cathinones, and some drugs only demonstrate minimal cross-reactivity, indicating that abuse of these substances may not be detected.

Several authors have also reported cross-reactivities of designer drugs when performing drug screens in case work for intoxications or deaths using a variety of types of immunoassays. Both BZP and TFMPP have been reported to cross-react in urine at varying concentrations with EMIT (enzyme multiplied immunoassay technique) d.a.u. (drug abuse assay) Amphetamine/ Methamphetamine II, Roche AbuScreen for Amphetamines, Syva EMIT II Plus for Amphetamines, and EMIT II Ecstasy [30, 31, 32]. Cross-reactivity has also been reported for phentermine in meconium (89% at 25 ng/g using Immunalysis ELISA for Amphetamine), AMT in urine and gastric contents (using Syva EMIT for Amphetamines), and mCPP in urine (positive at 5000- 7500 ng/mL using EMIT II Ecstasy) [32, 33, 34]. Others have reported that drugs such as DOB, 5-MeO-DiPT, 2C-T-4, and mephedrone test negative on screens for amphetamine, methamphetamine, or MDMA [35, 36, 37, 38]. Most recently, Torrance *et al.* reported cross-reactivity of mephedrone with a methamphetamine-based Immunalysis ELISA kit when investigating four fatalities [7]. The cross-reactivities ranged from 1-3% in urine and blood, yet no cross-reactivity was demonstrated with the amphetamine assay up to 5000 ng/mL. With regard to "bath salts", cross-reactivity has been observed with butylone on the Microgenics CEDIA Amphetamines/Ecstasy immunoassay [39] and well as with MDPV towards the PCP (phencyclidine) kit [40].

While large-scale cross-reactivity studies have become more prominent as the number of designer drugs on the market increases, one group, Petrie *et al*., took a different approach to these experiments by utilizing computational tools to predict cross-reactivity [41]. By evaluating two-dimensional and three-dimensional molecular structures, they were able to predict cross-reactivities of an additional 261 amphetamine-like compounds. This type of specialized methodology allows laboratories to investigate new entities and understand how they might behave in their current immunoassay screens. However, additional cross-reactivity studies are still necessary to corroborate the data generated by such computational techniques.

3. Statement of hypothesis or rationale for the research: Based on the above data, there is a critical need in the field of forensic toxicology for reliable screening assays for multiple designer drugs, in addition to analytical methods optimized for comprehensive screening and confirmation of such drugs in a variety of human specimens for both ante- and post-mortem investigation. The major goal of the present study was to evaluate the performance of commercially available screening immunoassays for detecting a wide range of designer drugs. Since each manufacturer is likely to employ different antibodies, specificity for individual drugs cannot always be predicted or compared among other types of immunoassays (e.g., EMIT), different matrices (i.e., meconium, whole blood, oral fluid), or different manufacturers. Regardless of any cross-reactivity that may occur, it is imperative that the forensic analytical toxicology community be made aware of the results, as screening techniques are limited for designer drugs, particularly the cathinone derivatives.

Two primary hypotheses were tested in this project: 1) Some amphetamine-like designer compounds would not be detected using conventional assays, even when multiple immunoassay platforms were utilized (*i.e.,* false negatives) and 2) some designer compounds would be detected

in assays that theoretically target only amphetamine and methamphetamine (*i.e.*, false positives). In a working toxicology lab, a negative result as in (1) would generally not be further analyzed or confirmed with other methods, with the result that the drugs may be overlooked. In contrast, a positive result as in (2) would trigger a confirmatory analysis, although an unknown designer drug would generally not be identified without proper reference standards or a comprehensive chromatographic method. A focus was placed on amphetamine or methamphetamine/MDMA reagents, as one or both of these types of assays are used in routine drug screens by a majority of labs. Since presumptive methods, like ELISA and EMIT, are the first line of screening methods for detecting drugs of abuse, it is necessary to understand how these important drugs can be detected, if at all, by currently available immunoassays, even where cross-reactivity is not expected.

II. Methods

Tasks: The present project as originally funded included two primary tasks: **Task 1** consisted of determination of the cross-reactivity of designer drugs in commercially available immunoassays. For this task, a total of 30 stimulant drugs in the phenethylamine, cathinone, tryptamine, and piperazine classes were assessed for cross-reactivity in 16 immunoassay (ELISA) platforms from four different manufacturers and also by EMIT analysis. Drugs were spiked into blank human serum (for ELISA) or urine (for EMIT) for these analyses. **Task 2** consisted of similar screening evaluation using a limited number of forensically relevant authentic blood and/or urine specimens, with comparison to screening results obtained using a validated LC-QQQ-MS method. This task was subsequently modified (see below) to include analysis of a series of blind spiked serum samples in addition to any available authentic specimens, and to add additional screening by a high-resolution MS-based approach (*i.e.,* LC-QTOF-MS).

Chemicals and materials: The following drugs were obtained from LipoMed (Cambridge, MA) as 1 mg/mL calibrated reference standards in solvent: 2C-B, (±)-3,4,5-TMA, (±)-4-methylethcathinone, (±)-butylone, (±)-cathinone, DMT, (±)-DOB, (±)-DOET, (±)-DOM, (±)-flephedrone, mCPP, (±)-MDPV, (±)-mephedrone, (±)-methcathinone, (±)-methedrone, (±)-methylone, (±)-N-ethylamphetamine, and TFMPP. The following drugs were obtained from Cerilliant (Round Rock, TX) as 1 mg/mL calibrated reference standards in solvent: *d*-amphetamine, *d*-methamphetamine, ketamine, methylphenidate, (±)-amphetamine, (±)-MDA, (±)-MDEA, (±)-MDMA, and (±)-methamphetamine. The following drugs were obtained from Grace Davison Discovery Sciences (Deerfield, IL) as 1 mg/mL calibrated reference standards in solvent: 2C-T-4, 2C-T-7, 2C-E, 2C-I, 5-MeO-DiPT, AMT, and BZP. An in-house standard of mephentermine, from powder, was available at a concentration of 1.02 mg/mL in methanol. The structures for each of the assay-targeted analytes and each of the analytes under investigation can be found in Tables 2 and 3. Methanol (GC$^{2®}$) was obtained from Honeywell Burdick & Jackson (Muskegon, MI). Dilution buffer (EIA buffer) and wash buffer (Wash Buffer Concentrate 10X) were obtained from Neogen Corporation (Lexington, KY). All other reagents were included in the individual immunoassay kits listed below.

2-Propanol (IPA, analytical grade), acetonitrile (Optima® LC-MS grade), ammonium formate, hydrochloric acid (HCl, analytical grade), glacial acetic acid (analytical grade), water (Optima® LC-MS grade), and methanol (Optima® LC-MS grade) were obtained from Fisher Scientific (Fair Lawn, NJ). Ammonium hydroxide (analytical grade) from Acros Organics (NJ), dichloromethane (analytical grade) from EMD Chemicals (Gibbstown, NJ), formic acid (Optima® LC-MS grade) from Fisher Scientific (Fair Lawn, NJ), and sodium phosphate monobasic monohydrate and dibasic heptahydrate (both analytical grade) from Acros (NJ) were also purchased for preparation of SPE reagents and mobile phases. Clean Screen® Extraction Columns (CSDAU, 200 mg; 10 mL) for solid-phase extraction were purchased from United Chem (Bristol, PA) for manual extraction performed on a positive pressure manifold from United Chem (Bristol, PA).

ELISA Reagents: Sixteen ELISA kits were obtained from four commercial manufacturers: Immunalysis Amphetamine Direct ELISA and Methamphetamine Direct ELISA (Pomona, CA); Neogen Amphetamine ELISA, Amphetamine Specific Forensic ELISA, Amphetamine Ultra Forensic ELISA, Benzylpiperazine Forensic ELISA, Ketamine Forensic ELISA,

Methylphenidate Forensic ELISA, Methamphetamine/MDMA Forensic ELISA, and Mephentermine Forensic ELISA (Lexington, KY); Randox MDPV ELISA and Mephedrone/Methcathinone ELISA (Co. Antrim, UK); and OraSure Technologies PCP Intercept®Micro-Plate EIA, Cotinine Serum Micro-Plate EIA, Amphetamine-Specific Serum Micro-Plate EIA, and Methamphetamine Intercept® Micro-Plate EIA (Bethlehem, PA). All of the antibodies were polyclonal in nature, with the exception of the PCP and Amphetamine-Specific kits from OraSure. Each kit consisted of 96-well microtiter plates coated with antibody for the targeted analyte, enzyme conjugate (3,3'5,5'-tetramethylbenzidine or TMB substrate solution), and an acid stop solution. A summary of the commercial immunoassays and reagents tested in this aspect of study can be found in Table 2.

EMIT Reagents: Two EMIT kits were obtained from Syva® (Siemens Healthcare Diagnostics; Newark, DE): EMIT® II Plus Ecstasy Assay and EMIT® II Plus Amphetamines Assay. The antibodies for the Ecstasy assay were polyclonal while those for the Amphetamines assay were monoclonal. Each kit supplied antibodies, drug-enzyme conjugate and all other necessary reagents. The EMIT® and EMIT® II Plus Ecstasy calibrators and controls were also purchased from Syva®. A summary of these reagents can be found in Table 3.

Serum and urine samples: For preparation of samples to be used in Task 1 studies, drug-free frozen serum, pooled from nine donors, was obtained from Utak Laboratories (Valencia, CA) and screened negative by ELISA for amphetamine, benzoylecgonine, ethanol, methamphetamine, morphine, oxazepam, phencyclidine, secobarbital, and THC-carboxy. This blank matrix was used for the preparation of controls as well as spiked samples. After thawing, it was stored at 4°C. Drug-free urine was obtained from a volunteer and used for the preparation of spiked samples. After collection, it was stored at 4°C.

For determination of cross-reactivity by ELISA, samples of drug-free serum (0.5 mL) were fortified with 50 µL of a methanolic spiking solution for analysis (see below). All calibrators, controls, and samples were subjected to a 1:4 (*i.e.,* 5-fold) dilution with buffer (EIA buffer) using a Hamilton Microlab® 500 Dual Syringe Diluter (Reno, NV). The dilution factor chosen was recommended by the manufacturer for forensic blood specimens. This helped to achieve uniformity and consistency between assays. For analysis by EMIT, samples of drug-free urine (1 mL) were fortified with 100 µL of a methanolic spiking solution for analysis (see below). The urine samples were not diluted prior to analysis, per manufacturer instructions.

For comparison of results using ELISA and LC-MS screening assays, 22 five mL serum samples were prepared and analyzed in a blind manner (*i.e.,* without analyst knowledge of drug identity or concentration). For this study, drug-free serum was spiked with known amounts of drug reference standards in methanolic solution. Samples were prepared that included single or mixtures of drugs and were prepared at a range of concentrations simulating those expected to be encountered in authentic specimens. The samples were given a unique ID number (1- 22) and frozen at -20°C until analysis by ELISA and LC-MS. Blind spiked sample compositions are summarized in Table 4.

In addition to the blind spiked samples, five authentic case (DUI/DWI) specimens were received from Palm Beach County Sheriff's Office (PBSO) Toxicology Unit. The whole blood specimens were stored in 10 mL grey top (fluoride and oxalate) vacutainers for 1-2 years mostly at room temperature but never opened. It was indicated that they were "positive for drugs" but none of the cathinone derivatives as far as the laboratory was aware. Any identifying or case information was removed from the labels prior to receiving them, and the specimens were relabeled as 7-001, 11-001, 11-002, 12-001, and 12-002 by PBSO. The specimens were stored at

4°C.

Sample extraction: For analysis by LC-MS, a solid phase extraction was performed to clean up the sample and isolate the drug. Serum samples (1 mL) were diluted with 2 mL of phosphate buffer (0.1 M, pH 6.0). The samples were gently vortexed and loaded onto a Clean Screen® SPE cartridge previously conditioned with 3 mL of methanol, 3 mL of water, and 1 mL of phosphate buffer. After extraction, the cartridges were sequentially washed with 1 mL of water, 1 mL of 0.1 M acetic acid, and then 1 mL of methanol. Pressure was applied until the cartridges were dry. Analytes were eluted slowly with 3 mL of elution buffer, which consisted of dichloromethane, IPA, and ammonium hydroxide (80:20:2 *v/v/v*). The eluates were acidified with 100 µL of 1% HCl in methanol before evaporation under nitrogen at 40°C in a TurboVap® LV by Caliper Life Sciences (Hopkinton, MA). When dry, the residue was reconstituted in 50 µL of mobile phase A (2 mM ammonium formate, 0.1% formic acid in water) and 5 µL were injected into the LC-MS system.

Instrumentation: ELISA was performed using a DSX® Four-Plate Automated ELISA processing System (Dynex Technologies; Chantilly, VA) operating Revelation version 6.15 software. The plates were read using a 450 nm filter. Test procedures were carried out according to manufacturers' instructions listed in the package inserts, as summarized in Table 5. All incubations were performed at ambient temperature. Wash buffer was diluted 10X with deionized (DI) water for use in the wash step (unless otherwise noted). Conjugates that were not "ready-to-use" were diluted according to the package inserts with the appropriate diluents provided from the manufacturer.

EMIT was performed using a V-Twin® analyzer (Siemens). The methods for qualitative analyses were downloaded from the manufacturer and carried out according to instructions. Daily calibrations were performed by running the appropriate calibrators for a 300 ng/mL cut-off. The calibration was validated by running negative and positive controls at the appropriate levels, per the kit inserts. Once the calibration was validated, urine specimens were analyzed.

LC-MS analysis was performed using two systems. Qualitative screening by LC-QQQ-MS employed an Agilent 1290 Infinity Binary Pump LC coupled to an Agilent 6460 triple quadrupole MS/MS with Jet Streaming technology and electrospray ionization (ESI) using Agilent MassHunter software. Separation occurred on an Agilent Zorbax Rapid Resolution HD Eclipse Plus C_{18} LC column (50 x 2.1 mm, 1.8 µm particle size). Data acquisition was performed in Dynamic MRM mode with positive ESI using one principal MRM transition for quantitation and one additional transition to serve as a qualifier for each analyte. Samples were also analyzed by high-resolution MS for confirmation and library matching. For this purpose, an Agilent 1290 Infinity Binary Pump LC coupled to an Agilent 6530 quadrupole time-of-flight (QTOF) was utilized. The same LC column as described above was used for separation. Data acquisition was performed in full-scan mode with positive ESI.

Preparation of drug standard solutions: For targeted analytes (Figure 1), 1 mg/mL methanolic reference standards were diluted with methanol for a final concentration of 100,000 ng/mL working stocks. These were diluted further to create spiking standards at concentrations of 2000, 1000, 500, 250, 100, 50, 25, and 10 ng/mL in methanol. For the analytes under investigation, 1 mg/mL reference standards were diluted with methanol (or appropriate solvent) for a final concentration of 100,000 ng/mL working stocks. These were diluted further to create spiking standards at concentrations of 50000, 25000, 12500, 6250, 3125, 1562, 781.2, 390.6, 195.3, 97.6 ng/mL in methanol (or solvent).

Cut-offs and controls: For an ELISA assay, the optimal range for cut-off values is typically provided by the kit's manufacturer. However, due to instrumental variation and varying matrices, it is important to determine the cut-off concentration from a dose response curve. There should be a displacement between 30 – 60% of B/B_0, where B = raw absorbance and B_0 = raw absorbance of the blank matrix, in order to demonstrate the greatest discrimination between positives and negatives. This level of displacement is consistent with the manufacturers' kit inserts and was used for determining the matrix-matched serum controls for the study. For each kit, the cut-off value was determined by preparing dose response curves in triplicate at decreasing concentrations by spiking 0.5 mL of serum with 50 µL of a methanolic spiking solution of the targeted analyte (10 – 100,000 ng/mL) to achieve concentrations in the range of 1 – 10,000 ng/mL. These samples were subjected to the dilution as previously described before analysis. The absorbance values at each concentration were averaged and displacement was calculated. The cut-off value with a displacement between 30 – 60% was chosen to be the "positive cut-off" for all future experiments with that kit. Negative controls and positive cut-off controls, made fresh daily, were run in duplicate with each kit during an experiment. A sample whose absorbance was greater than or equal to 1.2 times the absorbance of the positive cut-off control was considered negative. A sample whose absorbance was less than or equal to the positive cut-off control was considered positive. A sample whose absorbance was between that of the positive cut-off control and 1.2 times the absorbance of the positive cut-off controls was considered "+/-" or indeterminate.

For the EMIT reagents, calibrators and controls were purchased from the manufacturer. To calibrate the Amphetamines assay for a 300 ng/mL cut-off, EMIT® Calibrator/Control Level 1 was used. To calibrate the Ecstasy assay for a 300 ng/mL cut-off, EMIT® Ecstasy Calibrator/Control Level 2 was used. For a negative control, EMIT® Calibrator/Control Level 0 was used for both assays. For positive controls, EMIT® Calibrator/Control Level 5 (2000 ng/mL) was used for Amphetamines and EMIT® Calibrator/Control Level 4 (1000 ng/mL) was used for Ecstasy.

Determination of cross-reactivity: In order to initially assess cross-reactivity via ELISA, 50 µL of each analyte of interest at 100,000 ng/mL was spiked into 0.5 mL of serum, in duplicate, for a final concentration of 10,000 ng/mL. This concentration level was chosen based on the cross-reactivity studies performed by the manufacturers as outlined in the package inserts. These samples were diluted as previously described before analysis. The absorbance values at each concentration were averaged. If a drug resulted in a "positive" on the DSX report at this concentration when compared to the positive cut-off level, a dose response curve was then prepared and analyzed to calculate the cross-reactivity. For these compounds, dose response curves were prepared in duplicate at decreasing concentrations by spiking 0.5 mL of serum with 50 µL of a spiking solution (97.6 – 100,000 ng/mL) to achieve concentrations in the range of 9.76 to 10,000 ng/mL.

In order to calculate the percent cross-reactivity, the percent binding (calculated as $[A_{sample}/A_{negative}]*100$) was determined for each analyte at each concentration tested [18]. From these values, the EC_{50} (effective concentration for 50% binding) was also calculated for each targeted analyte and each analyte of interest. The concentration of each analyte of interest that produced an absorbance reading closest to that of the positive cut-off control was also calculated. This value is represented by the $A_{sample}/A_{negative}$ with a ratio closest to 1. The positive cut-off level was then divided by the concentration of each analyte with the same absorbance value and expressed as a percent, representing the percent cross-reactivity [27]. For terminology purposes, "false positive" is used for a compound of interest which exhibits a positive result by the DSX

when compared to the positive cut-off control.

In order to initially assess cross-reactivity via EMIT, 50 µL of each analyte of interest at 100,000 ng/mL was spiked into 0.5 mL of urine, in duplicate, for a final concentration of 10,000 ng/mL. The samples were analyzed after calibration was validated with appropriate controls. If a drug resulted in a "positive" on the V-Twin report at this concentration when compared to the cut-off level, a dose response curve was then prepared and analyzed to calculate cross-reactivity. For these compounds, dose response curves were prepared in duplicate at decreasing concentrations by spiking 0.5 mL of urine with 50 µL of a spiking solution (97.6 – 100,000 ng/mL) to achieve concentrations in the range of 9.76 – 10,000 ng/mL. In order to calculate the percent cross-reactivity, the concentration of each analyte of interest that produced an absorbance reading equivalent to that of the 300 ng/mL cut-off control was used (as described for the ELISA calculations above).

LC/MS conditions: Chromatographic separation occurred with gradient elution at a flow rate of 0.5 mL/min using 2 mM ammonium formate/0.1% formic acid in water as mobile phase A and acetonitrile/water (90:10 *v/v*) with 0.1% formic acid as mobile phase B. The gradient was as follows: 5% B up to 35% B in 6 minutes as the analytical run, followed by a 30 s ramp up to 95% B and then a 1 minute hold for clean-up before a 3.5 minute re-equilibration at 5% B. The analytical column was kept at a temperature of 40°C in a thermostatted column compartment during separation.

Source parameters for QQQ-MS were as follows: gas temperature 320°C; gas flow 8 L/min; nebulizer 27 psi; sheath gas heater 380°C; sheath gas flow 12 L/min; capillary voltage 3,750 V; and charging voltage 500 V. For increased sensitivity, a fully validated method previously published by the authors was utilized [42]. The method parameters are summarized in Table 6. Data were acquired in Dynamic MRM mode with two transitions per analyte and compounds were identified using Agilent MassHunter Qualitative Analysis software. Compounds were identified qualitatively by examining retention time and ion ratios for both transitions.

Source parameters for QTOF-MS analysis were as follows: gas temperature 320°C; gas flow 8 L/min; nebulizer 27 psi; sheath gas heater 380°C; sheath gas flow 12 L/min; capillary voltage 3,750 V; nozzle voltage 500 V; fragmentor 125; skimmer 65; octapole RF peak 750. Agilent MassHunter Qualitative Analysis software was employed for the identification of analytes. An in-house library with exact mass data was used for confirmation of the analytes. Retention time, as known from the QQQ method, was also considered when making matches. A software score of 90 or greater was considered a match if there was also minimal difference between the actual and expected mass.

III. Results

1. Task 1:

Establishing cut-off values: For each ELISA reagent, the displacement was calculated for each level of targeted analyte in the dose response curve. The concentration with a displacement value from 30-60% was chosen as the cut-off and used as positive cut-off controls for future experiments. A summary of these concentrations can be found in Table 2. An example of a dose response curve used for the determination of a cut-off value (for Neogen Ketamine) can be found in Figure 4. The values ranged from 1.25-100 ng/mL and were comparable to those cited in the package inserts. While some of these levels were quite low, the assays are not quantitative and can only presumptively identify a class of compounds. An analytical method with lower detection limits (such as LC-MS/MS) is recommended for confirmation or quantification of such compounds. For the EMIT reagents, there were several cut-off levels available among the various levels of EMIT® calibrators and controls. For both the Amphetamines and Ecstasy kits, the lowest level (300 ng/mL) was chosen.

Cross-reactivity for ELISA: The analytes of interest which did not generate a positive result for a specific reagent at 10,000 ng/mL were not further analyzed for that kit. The cut-off equivalent concentration, percent cross-reactivity, and EC_{50} for these analytes were calculated using the value at that level. Compounds which did indicate a positive result at 10,000 ng/mL were further analyzed down to concentrations as low as 10 ng/mL or until a negative result was produced. The dose response curves for these analytes were constructed (% binding vs. analyte concentration) in order to visually examine the cross-reactivity as well as the EC_{50}. Detailed results for individual platforms are presented in Tables 7-10 and summarized for all 16 reagents in Table 11.

Several kits did not exhibit cross-reactivity with any of the analytes of interest; Neogen Ketamine, Neogen Methylphenidate, OraSure PCP, and OraSure Cotinine. This finding was not unexpected, due to the structural differences between the analytes targeted by the kit and those under investigation here. The Neogen BZP kit demonstrated minimal cross-reactivity with MDEA, MDMA, and DMT at concentrations (5,000 to 10,000 ng/mL) which most likely would not be encountered in an authentic case. The cross-reactivity values for these compounds were less than 0.5% and are probably not significant. As reported in the manufacturer package inserts, amphetamine and/or methamphetamine were without any cross-reactivity, consequently positives from similar compounds to the amphetamines would not be anticipated. As mephentermine is structurally similar to methamphetamine, it was not a surprise that methamphetamine and MDMA demonstrated cross-reactivity at concentrations as low as 250 and 200 ng/mL, respectively, with cross-reactivity values of 4% and 5%, respectively. These concentrations are well within the range of those typically encountered in forensic specimens. The Neogen Mephentermine reagent also demonstrated minimal cross-reactivity with MDEA and ethylamphetamine, analytes that are also structurally similar. This cross-reactivity is also likely to be of less significance, since the positive results occurred at relatively high concentrations; 1,250 and 1,750 ng/mL, respectively.

Upon investigation of the amphetamine-targeting kits, it became apparent that these reagents were quite selective. The Immunalysis Amphetamine, Neogen Amphetamine Specific, and OraSure Amphetamine Specific reagents all produced positive test results for MDA and AMT at concentrations between 10-150 ng/mL, depending on the reagent. The extensive cross-reactivity

with MDA (90-250%) was expected, per the manufacturers' data. The cross-reactivity with AMT (30-120%) was not entirely surprising, given the cross-reactivity reported by Boland, et al. for the same compound in post-mortem urine and gastric contents when analyzed by EMIT amphetamine immunoassay [33]. The other two reagents, Neogen Amphetamine and Neogen Amphetamine Ultra, were less specific and demonstrated cross-reactivity with methamphetamine, MDEA, MDMA, ethylamphetamine, mCPP, and AMT in the range of 10-1,250 ng/mL. The results for methamphetamine and MDMA were consistent with those reported in the package inserts. MDEA and ethylamphetamine, both structurally similar to amphetamine, were not included in the manufacturer's data but the results appeared reasonable given those for MDMA. The cross-reactivity towards AMT was also not unexpected, given the results from the more specific amphetamine kits. The most remarkable result was that for mCPP, with cross-reactivity noted at concentrations of 150 ng/mL for both of these kits, resulting in cross-reactivity values of 32%. Without additional information regarding the specific antibody used in the kits (*e.g.*, hapten and carrier used for immunization, method of purification, etc.), it is difficult to explain this phenomenon. However, it is corroborated by the fact that TFMPP, similar in structure to mCPP, also demonstrated cross-reactivity at 2,500 ng/mL.

After examining the analytes of interest by methamphetamine-based reagents, it was evident that the results were comparable to those using kits targeting amphetamine, except with regard to the cathinone derivatives. The Immunalysis Methamphetamine, Neogen Methamphetamine/MDMA, and OraSure Methamphetamine kits displayed positive test results for MDEA, MDMA, and ethylamphetamine at low concentrations, with cross-reactivities of between 15 and 250%. While cross-reactivity was less than 2% for the cathinone derivatives using the Immunalysis or Neogen methamphetamine reagents, with positive test results at levels as low as 1,250 ng/mL, the OraSure assay demonstrated greater cross-reactivity for this class of compounds. Positive test results for mephedrone, methcathinone, methylone, 4-MEC, flephedrone, butylone, and methedrone were still observed at concentrations as low as 40-450 ng/mL, with cross-reactivity values in the range of 2-25%. While these findings indicate that the OraSure methamphetamine reagents are less specific than those from Immunalysis or Neogen, they also demonstrate that this assay kit may be a viable screening tool for presumptively detecting "bath salts" in biological fluids at concentrations that can be encountered in forensic specimens, without necessarily targeting overdose levels. As the OraSure kit is designed for screening in oral fluid, its use in screening other matrices may require additional validation.

While the previously described assays targeted amphetamine, methamphetamine, or other commonly encountered drugs, the Randox reagents were specifically designed to detect "bath salts" or cathinone derivatives. The Randox MDPV kit was extremely selective, with only butylone demonstrating cross-reactivity at levels as low as 150 ng/mL. Since the MDPV reagent did not produce positive test results with other cathinone derivatives, it can be hypothesized that the side chain on the α-carbon of MDPV may behave similarly to that of butylone. The Randox Mephedrone/Methcathinone kit was found to be less specific, as the other cathinone derivatives were still positive by the DSX at 150 ng/mL when compared to the positive cut-off control. Alternatively, the Mephedrone/Methcathinone kit did not demonstrate cross-reactivity towards MDPV, which might indicate that the activity would be hindered by the nitrogen-containing ring system on MDPV. This assay, however, did not demonstrate cross-reactivity towards other phenethylamines. While decomposed specimens were not evaluated in the present study, the Randox Mephedrone/Methcathinone assay may be beneficial as a screening tool for targeting "bath salts", as putrefactive amines may not interfere due to the high selectivity of the reagents.

Cross-reactivity for EMIT: The analytes of interest which did not generate a positive result for a specific reagent at 10,000 ng/mL were not further analyzed for that kit. The cut-off equivalent concentration and percent cross-reactivity were calculated using the value at that level. Compounds which did indicate a positive result at 10,000 ng/mL were further analyzed down to concentrations as low as 10 ng/mL or until a negative result was produced. The dose response curves for these analytes were constructed in order to examine the cross-reactivity. These data are summarized in Table 12.

The values for cross-reactivity for the compounds of interest were consistent with literature and the package inserts. The Amphetamines kit uses *d*-methamphetamine as the cut-off control, so the results for amphetamine were to be expected. The cross-reactivity with MDA, MDEA, and MDMA are comparable to those in the kit insert. From the behavior of AMT in the serum ELISAs, the reactivity of AMT was not unexpected, although it was not very high. With regard to the Ecstasy kit, the cross-reactivities for MDA and MDEA were comparable to those stated in the package insert. Surprisingly, butylone exhibited some cross-reactivity down to 4,000 ng/mL, which may indicate the level at which antibody binding occurs. However, the cathinone derivatives remained undetected by these reagents at high concentrations and would not be expected to be identified in urine by either of these reagents.

2. Task 2:

Procurement of authentic specimens for screening analysis: Task 2 of this project was originally formulated to consist of validation of immunoassay data using a series of authentic casework specimens, with a milestone of screening a minimum of 20 DUI/DUID/post-mortem specimens by immunoassay and an LC-MS based analytical method. At the time of this report, only five case samples were obtained. For this reason, a change in the approach for Task 2 became necessary due to difficulties in securing additional authentic case samples for validation of our immunoassay results with the four commercial platforms. Changes in Florida law regarding use of post-mortem specimens for research activities following initial funding of the project led to complications in obtaining such specimens from our project collaborators at Miami-Dade Medical Examiners Toxicology Laboratory. Task 2 was therefore modified in discussion with the NIJ Program Manager to include the use of blinded spiked blank human serum specimens for testing in addition to authentic case specimens.

Comparative screening of blind spiked samples by ELISA, LC-QQQ-MS, and LC-QTOF-MS: The twenty-two spiked blinded serum samples were thawed and screened by all 16 ELISA reagents as listed in Table 2. The samples were diluted as described above and analyzed against fresh positive and negative serum controls. The results are shown in Table 13. All of the samples screened positive for cotinine using the OraSure reagent. The blank serum used for the preparation of the controls was different than the lot of blank serum used to prepare the blind spiked specimens and therefore likely contained higher levels of the nicotine metabolite. Since this reagent did not exhibit any cross-reactivity towards the analytes of interest during the initial stages of this study, it was determined that the positive reactions are from the matrix alone and not any of the drugs that may have been added. Several samples gave indeterminate results, as indicated by "±" in the table.

Spiked samples were extracted by SPE as described under Methods. The results obtained by LC-QQQ-MS confirmed many of the "false-positives" obtained during the ELISA screening of

these samples. For example, the ELISA reagents targeting methamphetamine gave positive results for samples that did not contain methamphetamine, (*i.e.,* samples 3, 4, 11, 14, and 15). The reagents targeting amphetamine gave positives for samples that did not contain amphetamine (*i.e.,* samples 3, 4, 6, 7, 8, 9, and 15). The MDPV kit successfully detected the analyte in sample 14 but gave indeterminate results for samples 3, 4, 8, and 15, which did not contain this compound. The Mephedrone/Methcathinone reagent was able to successfully detect cathinone derivatives in 10 out of the 11 samples which contained such analytes. The other case, sample 20, was determined to contain flephedrone at a low concentration that was otherwise undetected by the immunoassays. Samples such as 13, 17, 18, and 19, which were assumed negative by immunoassay, were determined to contain 2C compounds when analyzed by LC-QQQ-MS.

The blind spiked samples were also analyzed by LC-QTOF-MS to confirm the results of the ELISA and LC-QQQ-MS analyses by means of high-resolution mass spectral library matching. The samples were analyzed in full-scan mode and compared to a full-scan library, while also considering retention time in identification of the analyte. The results are summarized in Table 14. Methamphetamine, amphetamine, and MDMA were not included in the mass spectral library and therefore could not be confirmed in samples 6, 7, 8, 9, and 11. There were no library matches found for samples 5 and 16, an expected result since these were negative samples. With the exceptions noted above, all of the compounds were successfully identified by QTOF-MS with high confidence, based on the high-resolution parent mass data obtained. With the exceptions of methedrone, butylone, and AMT, all of the compounds identified by the library had ID scores of at least 90 as provided by the Agilent MassHunter software.

The five authentic case specimens were screened by all 16 ELISA reagents. The samples were diluted as described above and analyzed against fresh positive and negative serum controls as well as whole blood controls (to ensure appropriate displacement with the different matrix). The results are shown in Table 115. None of the samples tested positive for any of the 16 reagents, except for 11-002, 12-001, and 12-002, which were positive for cotinine. Since this reagent did not exhibit any cross-reactivity towards the analytes of interest during the initial stages of this study, it was determined that the positive reactions were most likely from nicotine use.

The PBSO samples were also analyzed by LC-QQQ-MS and LC-QTOF-MS to confirm the results of the ELISA by means of targeted MRM as well as high-resolution mass spectral library matching. By LC-QQQ, the samples were analyzed using a dynamic MRM method which matches based on retention time and two ion transitions for 32 designer cathinone compounds. By LC-QTOF, the samples were analyzed in full-scan mode and compared to an in-house designer drug library and database as well as a forensic toxicology library and database supplied by Agilent Technologies. A library search provides matching based on spectral data while a database search provides matching based on mass. All five cases were negative for all drugs using the targeted LC-QQQ method. The results from LC-QTOF are summarized in Table 126. The positive cotinine ELISA results were confirmed for 11-002, 12-001, and 12-002 by the identification of cotinine and/or 3-hydroxycotinine, indicative of nicotine use. None of the targeted analytes of this research were identified by the in-house designer drug library or database. This is consistent with the negative ELISA results for the amphetamine-type compounds. Other drugs and their metabolites were detected as summarized in the table. However, these results are presumptive as these analytes were not a part of this study and reference standards were not readily available to confirm retention time or the library results.

IV. Conclusions

1. Discussion of findings: In this comprehensive study, thirty designer drug entities from the phenethylamine, tryptamine, and piperazine structural classes were evaluated against sixteen different commercial ELISA reagents and two commercial EMIT reagents in order to determine cross-reactivity. Since few assays are currently available that target these analytes, particularly the "bath salts", it was important to understand how such drugs may react, especially in presumptive screens. The first hypothesis proposed in the present investigation, *i.e.,* that some novel drugs would not be detected in conventional screening immunoassays, was confirmed. For example, cathinone derivatives and other designer drugs outside the realm of the traditional phenethylamines, such as amphetamine, methamphetamine, and MDMA were not routinely detected. This observation demonstrates that forensic toxicological screening approaches will not be able to solely rely on immunoassays, at least those currently available in the commercial marketplace.

The second hypothesis, *i.e.,* that some designer compounds would be detected in assays that target only amphetamine and methamphetamine, was also confirmed, although cross-reactivity with untargeted drugs was generally limited. MDA, MDMA, ethylamphetamine, and AMT demonstrated cross-reactivity at low concentrations, but results were consistent with those published by the manufacturer or as reported in the literature. Cross-reactivity towards the cathinone derivatives was also found to be limited. However, the cathinone derivatives did demonstrate cross-reactivity at low concentrations (<150 ng/mL) when analyzed against the Randox Mephedrone/Methcathinone kit. While this reagent seemed less selective, there was no cross-reactivity with other amphetamine-like compounds. This finding suggests that the Randox kit may be useful for detecting a wide range of "bath salts" in post-mortem specimens, without the usual interference from putrefactive amines formed during decomposition. Overall, a majority of the kits analyzed, particularly those targeting phenethylamines, did not exhibit cross-reactivity with the compounds of interest, particularly the cathinone derivatives.

2. Implications for policy and practice: An important conclusion from these data is that current immunoassay-based screening methods may not be ideal for presumptively identifying most designer drugs, including the "bath salts". Laboratories should be aware of the issue of cross-reactivity (or the lack thereof) when performing routine screens so that these types of compounds are not overlooked. While oral fluid may not be a commonly analyzed matrix, the results obtained for the cross-reactivity of the cathinone derivatives in serum by an oral fluid specific reagent (*i.e.,* the OraSure Methamphetamine kit) suggests its possible use for detecting such compounds. However, this would require extensive validation for this matrix and would not necessarily be applicable to every cathinone derivative.

Recently, there has been a trend toward the introduction of new immunoassays with specificity for individual designer drugs or groups of drugs, a development that can, at least to some extent, help address this problem. Alternatively, as more laboratories move towards LC-MS/MS as an in-house analytical tool, screening methods for such analytes will likely gravitate towards higher specificity approaches, in particular high-resolution, high mass accuracy MS. More advanced analytical techniques, such as LC-MS, are required for the identification of these compounds, as demonstrated by the LC-QQQ and LC-QTOF analyses. Toxicology labs should and will continue to move towards LC-MS or other advanced techniques for the detection of these compounds in routine screenings of biological specimens.

There are some limitations to the data produced in this study with regard to generalization to

the forensic toxicological screening process. For example, the study only examined 30 drugs, of which only eight were cathinone derivatives. In addition, no synthetic cannabinoids were included in the investigation. Many more compounds exist in the "designer drug" universe, and novel entities continue to be synthesized. Consequently, the current research cannot be considered comprehensive or complete with regard to all potential designer drug entities. Another limitation is that there are several commercially available screening platforms that were not evaluated in the study, and new assays with enhanced designer drug specificity continue to be introduced. These also need to be systematically evaluated with respect to drug cross-reactivity. One problem that was encountered in the present study, which necessitated a change in protocol, was the lack of availability of authentic specimens for comparative screening by immunoassay and LC-MS based methods. However, it is believed that the use of blind spiked specimens as an alternative approach was successful in achieving the original goals of Task 2 of the project.

3. Implications for further research: As noted above, future cross-reactivity studies should include evaluation of additional individual designer drugs, drug classes, and commercial immunoassays. As the designer drugs in the market become more diversified, additional analytes may need to be examined for cross-reactivity. While computational modeling can help in predicting cross-reactivity [41], actual screening experiments in working laboratories are necessary to confirm these predictions. In addition, future research should include similar cross-reactivity studies with large numbers of authentic forensic specimens, in order to provide insight into the effects of individual variability in sample matrix characteristics and possible drug-drug interactions that could influence assay cross-reactivity.

Despite the utility and widespread use of immunoassay-based screening in forensic toxicology, an important conclusion from these studies is that current screening methods may not be ideal for presumptively identifying the universe of designer drugs. The trend toward the introduction of new immunoassays with specificity for individual designer drugs or groups of drugs is a development that can, at least to some extent, help alleviate this problem. However, it is unreasonable to expect, in view of the time and effort necessary to produce them, that assay probes specific for each of the hundreds of individual designer drug entities will be available any time soon. Alternatively, as more laboratories move towards LC-MS/MS as an in-house analytical tool, screening methods for such analytes will likely gravitate towards higher specificity approaches, in particular high-resolution, high-mass-accuracy MS. However, despite recent advances in designer drug analysis by mass spectrometry, currently available MS-based screening methods are generally limited to analysis of up to several dozen relevant drugs, often from only one of the two primary designer drug classes (*i.e.,* amphetamine-like stimulants and synthetic cannabinoids). Consequently, there is a great need for research to validated new MS-based methods for screening and confirmation of the many hundreds of designer drugs potentially present in forensic toxicological specimens, in addition to methods capable of detecting and identifying novel compounds.

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VI. Dissemination of Research Findings

Publication:

Swortwood, M.J., Hearn, W.L., and DeCaprio, A.P. (2013). Cross-reactivity of designer drugs including cathinone derivatives in commercial enzyme-linked immunosorbent assays. *Drug Testing Anal.*, Ahead of print; DOI: 10.1002/dta.1489.

Presentations:

Swortwood, M.J., Boland, D.M., and DeCaprio, A.P. (2012). Designer drugs analysis in the United States. *Invited talk presented at the Forensic and Clinical Toxicology Association 2012 Scientific Workshop*; Hobart, Tasmania, September 28.

Swortwood, M.J., Hearn, W.L., and DeCaprio, A.P. (2013). Cross-reactivity of cathinone derivatives and other designer drugs in commercial enzyme-linked immunosorbent assays. *American Academy of Forensic Sciences 65th Annual Meeting,* Washington, DC; February 18-23.

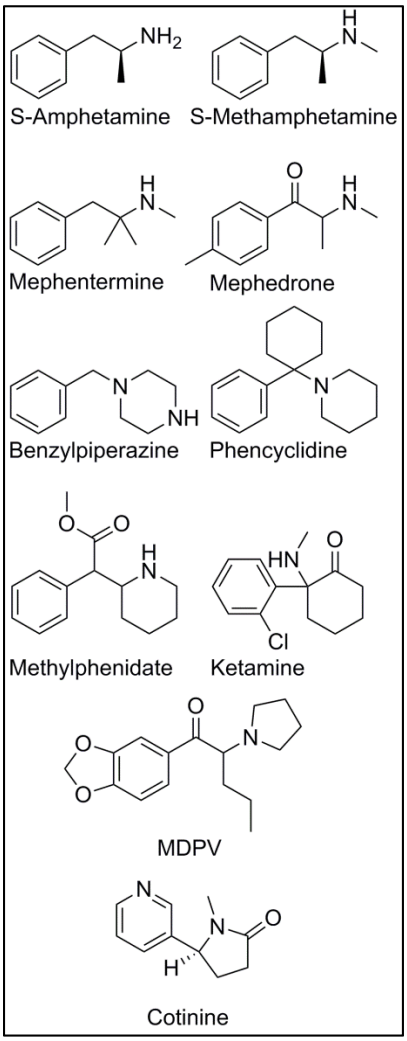

Figure 1: Immunoassay Target Compounds.

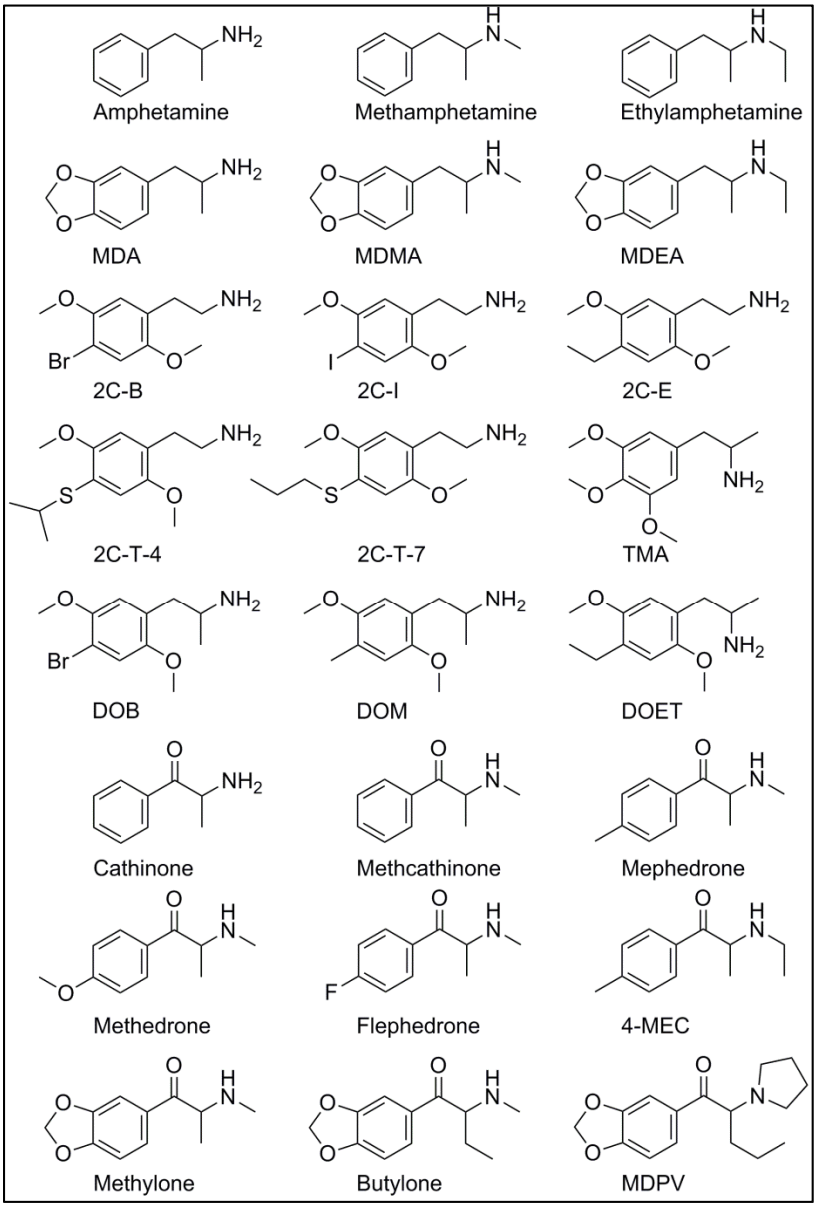

Figure 2: Designer Drugs of Interest: Phenethylamines.

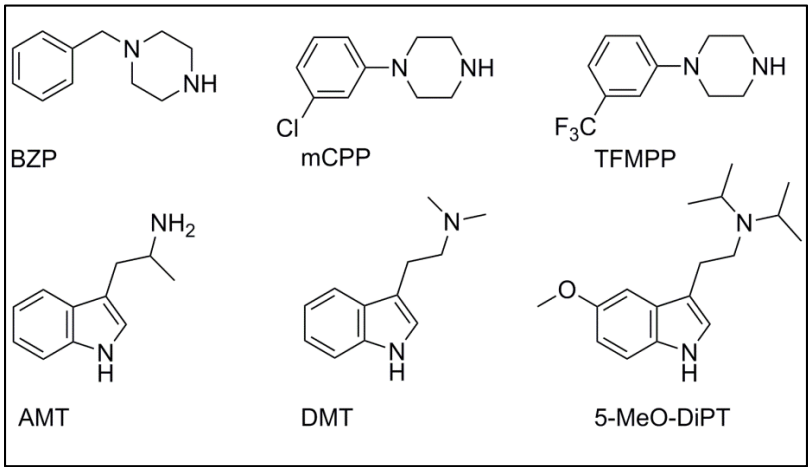

Figure 3: Designer Drugs of Interest: Piperazines and Tryptamines.

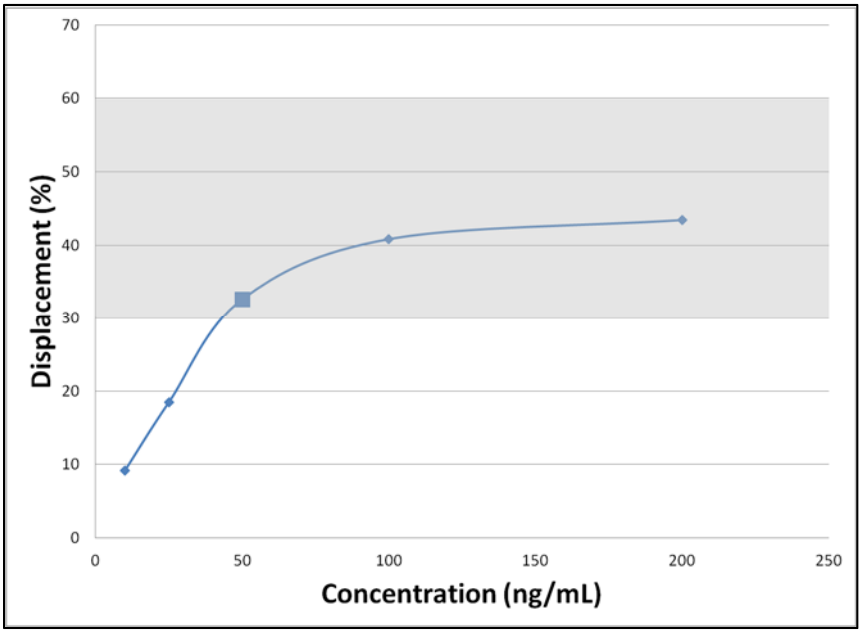

Figure 4: Dose-response curve for determining cut-off value for Neogen Ketamine ELISA, showing displacement (%) versus concentration (ng/mL). The ideal range for percent displacement is shaded. The data point for the chosen cut-off level is indicated by ■.

Table 1: Abbreviations for Designer Drug Analytes Targeted in the Study.

Abbreviation or Common Name	Chemical Name
2C-B	2,5-dimethoxy-4-bromophenethylamine
2C-E	2,5-dimethoxy-4-ethylphenethylamine
2C-I	2,5-dimethoxy-4-iodophenethylamine
2C-T-4	2,5-dimethoxy-4-(i)-propylthiophenethylamine
2C-T-7	2,5-dimethoxy-4-(n)-propylthiophenethylamine
4-FMC, Flephedrone	4-fluoromethcathinone
4-MEC	4-methylethcathinone
4-MMC, Mephedrone	4-methylmethcathinone
5-MeO-DiPT	5-methoxy-diisopropyltryptamine
AMT	α-methyltryptamine
bk-MBDB, Butylone	3,4-methylenedioxyethcathinone
bk-MDMA, Methylone	3,4-methylenedioxymethcathinone
bk-PMMA, PMMC, Methedrone	4-methoxymethcathinone
BZP	benzylpiperazine
Cathinone	α-aminopropiophenone
DMT	dimethyltryptamine
DOB	2,5-dimethoxy-4-bromoamphetamine
DOET	2,5-dimethoxy-4-ethylamphetamine
DOM	2,5-dimethoxy-4-methylamphetamine
mCPP	3-chlorophenylpiperazine
MDA	3,4-methylenedioxyamphetamine
MDEA	3,4-methylenedioxyethylamphetamine
MDMA, Ecstasy	3,4-methylenedioxymethamphetamine
MDPV	3,4-methylenedioxypyrovalerone
Methcathinone	2-methylaminopropiophenone
TFMPP	3-trifluoromethylphenylpiperazine
TMA	3,4,5-trimethoxyamphetamine

Table 2: Commercial ELISA Kits and Reagents.

Manufacturer	Kit	Targeted Analyte[a]	Type of Antibody	Cut-Off[b] (ng/mL)
Immunalysis	Amphetamine Direct ELISA	d-Amphetamine	Polyclonal	25
	Methamphetamine Direct ELISA	d-Methamphetamine	Polyclonal	25
Neogen	Amphetamine ELISA	d-Amphetamine	Polyclonal	50
	Amphetamine Specific Forensic ELISA	d-Amphetamine	Polyclonal	50
	Amphetamine Ultra Forensic ELISA	d-Amphetamine	Polyclonal	50
	Benzylpiperazine Forensic ELISA	Benzylpiperazine	Polyclonal	25
	Ketamine Forensic ELISA	Ketamine	Polyclonal	50
	Methylphenidate Forensic ELISA	Methylphenidate	Polyclonal	10
	Methamphetamine/MDMA Forensic ELISA	d-Methamphetamine	Polyclonal	25
	Mephentermine Forensic ELISA	Mephentermine	Polyclonal	10
Randox	MDPV ELISA	MDPV	Polyclonal	10
	Mephedrone/Methcathinone ELISA	Mephedrone	Polyclonal	1.25
OraSure	PCP Intercept® Micro-Plate EIA	Phencyclidine	Monoclonal	20
	Cotinine Serum Micro-Plate EIA	Cotinine	Polyclonal	100
	Amphetamine-Specific Serum Micro-Plate EIA	d-Amphetamine	Monoclonal	50
	Methamphetamine Intercept® Micro-Plate EIA	d-Methamphetamine	Polyclonal	10

[a] Kit's targeted analyte used for controls.
[b] Experimentally determined. See Materials and Methods.

Table 3: Commercial EMIT Kits and Reagents.

Manufacturer	Kit	Type of Antibody	Cut-Off[a] (ng/mL)
Syva	EMIT II Plus Amphetamines	Monoclonal	300
	EMIT II Plus Ecstasy	Polyclonal	300

[a] Chosen from Manufacturer. See Materials and Methods.

Table 4: Composition of Blind Spiked Samples for Task 2 Screening.

ID	Drug(s)	nominal concentration (ng/mL)
1	methylone	20
2	methylone	20
3	ethylamphetamine	100
4	ethylamphetamine	100
5	no spike	0
6	methamphetamine + methedrone	100 + 100
7	methamphetamine + methylone	100 + 100
8	methamphetamine + 2C-I	100 + 100
9	methamphetamine + MDMA	120 + 120
10	methylone + flephedrone	120 + 120
11	amphetamine + mCPP	100 + 100
12	methylone + 5-MeO-DiPT	100 + 100
13	2C-I + 2C-T-7	100 + 100
14	MDPV + mephedrone	100 + 100
15	AMT + butylone + ethylamphetamine	100 + 100 + 100
16	no spike	0
17	2C-I	20
18	2C-I	60
19	2C-I	200
20	flephedrone	20
21	flephedrone	60
22	flephedrone	200

Table 5: Test Procedures for ELISA Analysis.

Manufacturer	Kit	Sample Volume (µL)	Conjugate Volume (µL)	Incubation Time (min)	No. of Wash Cycles	Wash Volume (µL)	Wash Solution	Substrate Volume (µL)	Incubation Time (min)	Stop Reagent Volume (µL)
Immunalysis	Amphetamine	10	100	60	6	350	DI water	100	30	100
	Methamphetamine	10	100	60	6	350	DI water	100	30	100
Neogen	Amphetamine	20	180	45	5	300	Wash buffer	150	30	50
	Amphetamine Specific	10	100	45	5	300	Wash buffer	100	30	100
	Amphetamine Ultra	10	100	45	5	300	Wash buffer	100	30	100
	Benzylpiperazine	20	50	45	5	300	Wash buffer	150	30	50
	Ketamine	20	100	45	5	300	Wash buffer	100	30	100
	Methylphenidate	20	100	45	5	300	Wash buffer	100	30	100
	Methamphetamine/MDMA	20	100	45	5	300	Wash buffer	100	30	100
	Mephentermine	20	180	45	5	300	Wash buffer	150	30	50
Randox	MDPV	50	75	60	6	300	Wash buffer	125	20	100
	Mephedrone/Methcathinone	25	100	60	6	300	Wash buffer	125	20	100
OraSure	PCP	50[a]	50	30	6	300	DI water	100	30	100
	Cotinine	10	100	30	6	300	DI water	100	30	100
	Amphetamine Specific	25	100	30	6	300	DI water	100	30	100
	Methamphetamine	25	100	30	6	300	Di water	100	30	100

[a] 50 µL OraSure Pre-Buffer added to wells after samples were dispensed.

Table 6: QQQ Acquisition Parameters for Dynamic MRM.

No.	Drug	Transitions[a]	CE (V)	Fragmentor (V)	t_R (min)
1	DOB	**274.01 → 256.9**	14	100	3.846
		274.01 → 228.9	10		
2	DOET	**224.3 → 207**	5	85	4.547
		224.3 → 91	49		
3	DOM	**210.3 → 193.1**	5	75	3.538
		210.3 → 165	13		
4	TMA	**226.3 → 209**	5	80	2.075
		226.3 → 91	45		
5	2C-B	**260.01 → 242.9**	4	90	3.403
		260.01 → 227.9	6		
6	2C-E	**210.3 → 193**	5	80	4.119
		210.3 → 163	25		
7	2C-I	**308.1 → 290.9**	9	90	3.906
		308.1 → 91	49		
8	2C-T-4	**256.4 → 239**	5	90	4.675
		256.4 → 197	17		
9	2C-T-7	**256.4 → 239**	9	85	4.959
		256.4 → 166.9	29		
10	MDA	**180.1 → 163**	4	70	1.658
		180.1 → 105	20		
11	MDEA	**208.14 → 163**	8	90	2.22
		208.14 → 105	24		
12	MDMA	**194.1 → 163**	8	85	1.849
		194.1 → 105	24		
13	Amphetamine	**136.11 → 91**	16	75	1.49
		136.11 → 119	4		
14	Methamphetamine	**150.13 → 91**	16	80	1.715
		150.13 → 119	4		
15	Ethylamphetamine	**164.11 → 91**	20	85	2.093
		164.11 → 119	8		
16	MDPV	**276.3 → 126**	25	130	3.383
		276.3 → 135	25		
17	Mephedrone	**178.25 → 160**	10	85	2.123
		178.25 → 144	30		

No.	Drug	Transitions[a]	CE (V)	Fragmentor (V)	t_R (min)
18	Cathinone	**150.2 → 132**	10	80	1.031
		150.2 → 117	22		
19	Methcathinone	**164.23 → 146**	10	85	1.196
		164.23 → 130	34		
20	Methedrone	**194.25 → 176**	10	80	1.745
		194.25 → 161	18		
21	4-MEC	**192.28 → 174.1**	10	95	2.482
		192.28 → 145	18		
22	Flephedrone	**182.21 → 164**	10	85	1.422
		182.21 → 148	34		
23	Methylone	**208.24 → 160**	14	80	1.397
		208.24 → 132	26		
24	Butylone	**222.26 → 174**	14	95	2.035
		222.26 → 204	10		
25	BZP	**177.11 → 91**	20	100	0.589
		177.11 → 65	50		
26	DBZP	**267.21 → 91**	32	125	3.52
		267.21 → 175	12		
27	mCPP	**197.11 → 153.9**	20	120	2.878
		197.11 → 118	36		
28	TFMPP	**231.11 → 188**	20	125	3.826
		231.11 → 118	44		
29	AMT	**175.2 → 158**	9	75	2.037
		175.2 → 143	25		
30	DMT	**189.11 → 58.1**	8	85	1.775
		189.11 → 144	16		
31	5-MeO-DMT	**219.3 → 58.1**	9	85	1.955
		219.3 → 174	9		
32	5-MeO-DiPT	**275.4 → 174**	17	100	3.627
		275.4 → 114.1	13		

Table 7: Cross-Reactivity Data for Amphetamine ELISA Reagents.

	Immunalysis			Neogen									OraSure		
	Amphetamine			Amphetamine			Amphetamine Specific			Amphetamine Ultra			Amphetamine Specific		
Drug	**C_{25}[a] (ng/mL)**	**Cross-Reactivity (%)**	**EC_{50} (ng/mL)**	**C_{50}[b] (ng/mL)**	**Cross-Reactivity (%)**	**EC_{50} (ng/mL)**	**C_{50}[b] (ng/mL)**	**Cross-Reactivity (%)**	**EC_{50} (ng/mL)**	**C_{50}[b] (ng/mL)**	**Cross-Reactivity (%)**	**EC_{50} (ng/mL)**	**C_{50}[b] (ng/mL)**	**Cross-Reactivity (%)**	**EC_{50} (ng/mL)**
(+)-Amphetamine	***25***	***100***	***18***	***50***	***100***	***200***	***50***	***100***	***100***	***50***	***100***	***10***	***50***	***100***	***70***
(±)-Methamphetamine	> 10,000	< 0.25	> 10,000	**< 10**	**> 500**	**10**	> 10,000	< 0.5	> 10,000	**< 10**	**> 500**	**15**	> 10,000	< 0.5	> 10,000
2C-E	> 10,000	< 0.25	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.5	> 10,000
(±)-DOET	> 10,000	< 0.25	> 10,000	> 10,000	< 0.5	> 10,000	5,000	1	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.5	> 10,000
(±)-DOM	10,000	0.25	6,750	> 10,000	< 0.5	> 10,000	5,000	1	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.5	> 10,000
(±)-TMA	5,000	0.5	6,750	> 10,000	< 0.5	> 10,000	2,500	2	10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.5	> 10,000
(±)-MDA	**< 10**	**> 250**	**< 10**	1,250	4	5,000	**78**	**64**	**100**	2,500	2	7,000	**56**	**89**	**64**
(±)-MDEA	7,000	0.36	6,500	**156**	**32**	**625**	> 10,000	< 0.5	> 10,000	**313**	**16**	**1,000**	> 10,000	< 0.5	> 10,000
(±)-MDMA	5,000	0.5	4,250	**156**	**32**	**1,250**	> 10,000	< 0.5	> 10,000	**625**	**8**	**2,500**	> 10,000	< 0.5	> 10,000
(±)-Ethylamphetamine	> 10,000	< 0.25	> 10,000	**< 10**	**> 500**	**< 10**	> 10,000	< 0.5	> 10,000	**< 10**	**> 500**	**19**	> 10,000	< 0.5	> 10,000
(±)-MDPV	> 10,000	< 0.25	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.5	> 10,000
(±)-Mephedrone	> 10,000	< 0.25	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.5	> 10,000
(±)-Cathinone	> 10,000	< 0.25	> 10,000	2,500	2	10,000	> 10,000	< 0.5	> 10,000	10,000	0.5	> 10,000	> 10,000	< 0.5	> 10,000
(±)-Methcathinone	> 10,000	< 0.25	> 10,000	1,250	4	5,000	> 10,000	< 0.5	> 10,000	4,250	1	4,500	> 10,000	< 0.5	> 10,000
(±)-Methylone	> 10,000	< 0.25	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.5	> 10,000
(±)-4-MEC	> 10,000	< 0.25	> 10,000	10,000	0.5	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.5	> 10,000
(±)-Flephedrone	> 10,000	< 0.25	> 10,000	1,250	4	> 10,000	> 10,000	< 0.5	> 10,000	10,000	0.5	> 10,000	> 10,000	< 0.5	> 10,000
(±)-Butylone	> 10,000	< 0.25	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.5	> 10,000
mCPP	> 10,000	< 0.25	> 10,000	**156**	**32**	**625**	> 10,000	< 0.5	> 10,000	**156**	**32**	**1,000**	> 10,000	< 0.5	> 10,000
(±)-Methedrone	> 10,000	< 0.25	> 10,000	10,000	0.5	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.5	> 10,000
5-MeO-DiPT	> 10,000	< 0.25	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.5	> 10,000
(±)-DOB	> 10,000	< 0.25	> 10,000	10,000	0.5	> 10,000	> 10,000	< 0.5	> 10,000	10,000	0.5	> 10,000	> 10,000	< 0.5	> 10,000
2C-B	> 10,000	< 0.25	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.5	> 10,000
DMT	> 10,000	< 0.25	> 10,000	5,000	1	> 10,000	> 10,000	< 0.5	> 10,000	5,000	1	> 10,000	> 10,000	< 0.5	> 10,000
BZP	> 10,000	< 0.25	> 10,000	5,000	1	> 10,000	> 10,000	< 0.5	> 10,000	3,000	1.67	10,000	> 10,000	< 0.5	> 10,000
AMT	**30**	**83**	**20**	**625**	**8**	**4,000**	**< 156**	**> 32**	**156**	1,250	4	4,500	**43**	**116**	**48**
2C-I	> 10,000	< 0.25	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.5	> 10,000
2C-T-7	> 10,000	< 0.25	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.5	> 10,000
TFMPP	> 10,000	< 0.25	> 10,000	2,500	2	10,000	> 10,000	< 0.5	> 10,000	2,500	2	> 10,000	> 10,000	< 0.5	> 10,000
2C-T-4	> 10,000	< 0.25	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.5	> 10,000

[a] Concentration of the drug that produces an absorbance reading equivalent to the 25 ng/mL cut-off of the targeted analyte.

[b] Concentration of the drug that produces an absorbance reading equivalent to the 50 ng/mL cut-off of the targeted analyte.

Analytes demonstrating high cross-reactivity are **highlighted and bolded**.

The target analytes for each reagent are ***italicized and bolded***.

Table 8: Cross-Reactivity Data for Methamphetamine/MDMA ELISA Reagents.

	Immunalysis			Neogen			OraSure		
	Methamphetamine			Methamphetamine/MDMA			Methamphetamine		
Drug	**C_{25}[a] (ng/mL)**	**Cross-Reactivity (%)**	**EC_{50} (ng/mL)**	**C_{25}[a] (ng/mL)**	**Cross-Reactivity (%)**	**EC_{50} (ng/mL)**	**C_{10}[b] (ng/mL)**	**Cross-Reactivity (%)**	**EC_{50} (ng/mL)**
(+)-Methamphetamine	***25***	***100***	***35***	***25***	***100***	***50***	***10***	***100***	***10***
(±)-Amphetamine	> 10,000	< 0.25	> 10,000	4,000	0.63	6,750	2,500	0.4	1,250
2C-E	> 10,000	< 0.25	> 10,000	> 10,000	< 0.25	> 10,000	> 10,000	< 0.1	> 10,000
(±)-DOET	> 10,000	< 0.25	> 10,000	> 10,000	< 0.25	> 10,000	> 10,000	< 0.1	> 10,000
(±)-DOM	> 10,000	< 0.25	> 10,000	> 10,000	< 0.25	> 10,000	> 10,000	< 0.1	> 10,000
(±)-TMA	> 10,000	< 0.25	> 10,000	> 10,000	< 0.25	> 10,000	> 10,000	< 0.1	> 10,000
(±)-MDA	5,000	0.5	6,000	1,250	2	2,000	**625**	**2**	**500**
(±)-MDEA	**35**	**71**	**40**	**156**	**16**	**313**	**10**	**100**	**< 10**
(±)-MDMA	**< 10**	**> 250**	**10**	***15***	***167***	***25***	**< 10**	**> 100**	**< 10**
(±)-Ethylamphetamine	**80**	**31**	**100**	**156**	**16**	**600**	**15**	**67**	**< 10**
(±)-MDPV	> 10,000	< 0.25	> 10,000	> 10,000	< 0.25	> 10,000	> 10,000	< 0.1	> 10,000
(±)-Mephedrone	1,250	2	2,500	2,500	1	9,000	**40**	**25**	**20**
(±)-Cathinone	> 10,000	< 0.25	> 10,000	> 10,000	< 0.25	> 10,000	> 10,000	< 0.1	> 10,000
(±)-Methcathinone	5,000	0.5	5,000	5,000	0.5	> 10,000	**300**	**3.33**	**150**
(±)-Methylone	2,500	1	4,000	5,000	0.5	> 10,000	**150**	**6.67**	**< 150**
(±)-4-MEC	1,250	2	1,250	2,500	1	> 10,000	**40**	**25**	**20**
(±)-Flephedrone	10,000	0.25	10,000	2,500	1	> 10,000	**450**	**2.22**	**250**
(±)-Butylone	10,000	0.25	10,000	5,000	0.5	> 10,000	**300**	**3**	**175**
mCPP	> 10,000	< 0.25	> 10,000	> 10,000	< 0.25	> 10,000	> 10,000	< 0.1	> 10,000
(±)-Methedrone	3,500	0.71	3,500	1,250	2	7,000	**150**	**6.67**	**60**
5-MeO-DiPT	> 10,000	< 0.25	> 10,000	> 10,000	< 0.25	> 10,000	> 10,000	< 0.1	> 10,000
(±)-DOB	> 10,000	< 0.25	> 10,000	> 10,000	< 0.25	> 10,000	> 10,000	< 0.1	> 10,000
2C-B	> 10,000	< 0.25	> 10,000	> 10,000	< 0.25	> 10,000	> 10,000	< 0.1	> 10,000
DMT	> 10,000	< 0.25	> 10,000	> 10,000	< 0.25	> 10,000	> 10,000	< 0.1	> 10,000
BZP	> 10,000	< 0.25	> 10,000	> 10,000	< 0.25	> 10,000	> 10,000	< 0.1	> 10,000
AMT	> 10,000	< 0.25	> 10,000	5,000	0.5	> 10,000	2,500	0.4	2,000
2C-I	> 10,000	< 0.25	> 10,000	> 10,000	< 0.25	> 10,000	> 10,000	< 0.1	> 10,000
2C-T-7	> 10,000	< 0.25	> 10,000	> 10,000	< 0.25	> 10,000	> 10,000	< 0.1	> 10,000
TFMPP	> 10,000	< 0.25	> 10,000	> 10,000	< 0.25	> 10,000	> 10,000	< 0.1	> 10,000
2C-T-4	> 10,000	< 0.25	> 10,000	> 10,000	< 0.25	> 10,000	> 10,000	< 0.1	> 10,000

[a] Concentration of the drug that produces an absorbance reading equivalent to the 25 ng/mL cut-off of the targeted analyte.

[b] Concentration of the drug that produces an absorbance reading equivalent to the 10 ng/mL cut-off of the targeted analyte.

Analytes demonstrating high cross-reactivity are **highlighted and bolded**.

The target analytes for each reagent are ***italicized and bolded***.

Table 9: Cross-Reactivity Data for Additional Neogen ELISA Reagents.

	Neogen											
	Benzylpiperazine			Ketamine			Methylphenidate			Mephentermine		
Drug	**C_{25}[a] (ng/mL)**	**Cross-Reactivity (%)**	**EC_{50} (ng/mL)**	**C_{50}[b] (ng/mL)**	**Cross-Reactivity (%)**	**EC_{50} (ng/mL)**	**C_{10}[c] (ng/mL)**	**Cross-Reactivity (%)**	**EC_{50} (ng/mL)**	**C_{10}[c] (ng/mL)**	**Cross-Reactivity (%)**	**EC_{50} (ng/mL)**
(±)-Amphetamine	> 10,000	< 0.25	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.1	> 10,000	> 10,000	< 0.1	> 10,000
(±)-Methamphetamine	10,000	0.25	10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.1	> 10,000	**250**	**4**	**400**
2C-E	> 10,000	< 0.25	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.1	> 10,000	> 10,000	< 0.1	> 10,000
(±)-DOET	> 10,000	< 0.25	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.1	> 10,000	> 10,000	< 0.1	> 10,000
(±)-DOM	> 10,000	< 0.25	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.1	> 10,000	> 10,000	< 0.1	> 10,000
(±)-TMA	> 10,000	< 0.25	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.1	> 10,000	> 10,000	< 0.1	> 10,000
(±)-MDA	> 10,000	< 0.25	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.1	> 10,000	10,000	0.1	> 10,000
(±)-MDEA	9,000	0.25	10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.1	> 10,000	1,250	0.80	7,000
(±)-MDMA	10,000	0.25	10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.1	> 10,000	**200**	**5**	**750**
(±)-Ethylamphetamine	> 10,000	< 0.25	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.1	> 10,000	1,750	0.60	3,000
(±)-MDPV	> 10,000	< 0.25	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.1	> 10,000	> 10,000	< 0.1	> 10,000
(±)-Mephedrone	> 10,000	< 0.25	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.1	> 10,000	> 10,000	< 0.1	> 10,000
(±)-Cathinone	> 10,000	< 0.25	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.1	> 10,000	> 10,000	< 0.1	> 10,000
(±)-Methcathinone	> 10,000	< 0.25	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.1	> 10,000	> 10,000	< 0.1	> 10,000
(±)-Methylone	> 10,000	< 0.25	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.1	> 10,000	> 10,000	< 0.1	> 10,000
(±)-4-MEC	> 10,000	< 0.25	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.1	> 10,000	> 10,000	< 0.1	> 10,000
(±)-Flephedrone	> 10,000	< 0.25	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.1	> 10,000	> 10,000	< 0.1	> 10,000
(±)-Butylone	> 10,000	< 0.25	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.1	> 10,000	> 10,000	< 0.1	> 10,000
mCPP	> 10,000	< 0.25	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.1	> 10,000	> 10,000	< 0.1	> 10,000
(±)-Methedrone	> 10,000	< 0.25	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.1	> 10,000	> 10,000	< 0.1	> 10,000
5-MeO-DiPT	> 10,000	< 0.25	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.1	> 10,000	> 10,000	< 0.1	> 10,000
(±)-DOB	> 10,000	< 0.25	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.1	> 10,000	> 10,000	< 0.1	> 10,000
2C-B	> 10,000	< 0.25	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.1	> 10,000	> 10,000	< 0.1	> 10,000
DMT	5,000	0.50	5,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.1	> 10,000	> 10,000	< 0.1	> 10,000
BZP	***25***	***100***	***35***	> 10,000	< 0.5	> 10,000	> 10,000	< 0.1	> 10,000	> 10,000	< 0.1	> 10,000
AMT	> 10,000	< 0.25	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.1	> 10,000	> 10,000	< 0.1	> 10,000
2C-I	> 10,000	< 0.25	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.1	> 10,000	> 10,000	< 0.1	> 10,000
2C-T-7	> 10,000	< 0.25	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.1	> 10,000	> 10,000	< 0.1	> 10,000
TFMPP	> 10,000	< 0.25	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.1	> 10,000	> 10,000	< 0.1	> 10,000
2C-T-4	> 10,000	< 0.25	> 10,000	> 10,000	< 0.5	> 10,000	> 10,000	< 0.1	> 10,000	> 10,000	< 0.1	> 10,000

[a] Concentration of the drug that produces an absorbance reading equivalent to the 25 ng/mL cut-off of the targeted analyte.

[b] Concentration of the drug that produces an absorbance reading equivalent to the 50 ng/mL cut-off of the targeted analyte.

[c] Concentration of the drug that produces an absorbance reading equivalent to the 10 ng/mL cut-off of the targeted analyte.

Analytes demonstrating high cross-reactivity are **highlighted and bolded**.

The target analytes for each reagent are ***italicized and bolded***.

Table 10: Cross-Reactivity Data for Additional Randox and OraSure ELISA Reagents.

	Randox						OraSure					
	MDPV			Mephedrone/Methcathinone			PCP			Cotinine		
Drug	**C_{10}[a] (ng/mL)**	**Cross-Reactivity (%)**	**EC_{50} (ng/mL)**	**$C_{1.25}$[b] (ng/mL)**	**Cross-Reactivity (%)**	**EC_{50} (ng/mL)**	**C_{20}[c] (ng/mL)**	**Cross-Reactivity (%)**	**EC_{50} (ng/mL)**	**C_{100}[d] (ng/mL)**	**Cross-Reactivity (%)**	**EC_{50} (ng/mL)**
(±)-Amphetamine	> 10,000	< 0.1	> 10,000	> 10,000	< 0.0125	> 10,000	> 10,000	< 0.2	> 10,000	> 10,000	< 1	> 10,000
(±)-Methamphetamine	> 10,000	< 0.1	> 10,000	5,000	0.0250	10,000	> 10,000	< 0.2	> 10,000	> 10,000	< 1	> 10,000
2C-E	> 10,000	< 0.1	> 10,000	> 10,000	< 0.0125	> 10,000	> 10,000	< 0.2	> 10,000	> 10,000	< 1	> 10,000
(±)-DOET	> 10,000	< 0.1	> 10,000	> 10,000	< 0.0125	> 10,000	> 10,000	< 0.2	> 10,000	> 10,000	< 1	> 10,000
(±)-DOM	> 10,000	< 0.1	> 10,000	> 10,000	< 0.0125	> 10,000	> 10,000	< 0.2	> 10,000	> 10,000	< 1	> 10,000
(±)-TMA	> 10,000	< 0.1	> 10,000	> 10,000	< 0.0125	> 10,000	> 10,000	< 0.2	> 10,000	> 10,000	< 1	> 10,000
(±)-MDA	> 10,000	< 0.1	> 10,000	> 10,000	< 0.0125	> 10,000	> 10,000	< 0.2	> 10,000	> 10,000	< 1	> 10,000
(±)-MDEA	> 10,000	< 0.1	> 10,000	10,000	0.0125	> 10,000	> 10,000	< 0.2	> 10,000	> 10,000	< 1	> 10,000
(±)-MDMA	> 10,000	< 0.1	> 10,000	2,500	0.05	5,000	> 10,000	< 0.2	> 10,000	> 10,000	< 1	> 10,000
(±)-Ethylamphetamine	> 10,000	< 0.1	> 10,000	> 10,000	< 0.0125	> 10,000	> 10,000	< 0.2	> 10,000	> 10,000	< 1	> 10,000
(±)-MDPV	***10***	***100***	***60***	> 10,000	< 0.0125	> 10,000	> 10,000	< 0.2	> 10,000	> 10,000	< 1	> 10,000
(±)-Mephedrone	> 10,000	< 0.1	> 10,000	***1.25***	***100***	***2.5***	> 10,000	< 0.2	> 10,000	> 10,000	< 1	> 10,000
(±)-Cathinone	> 10,000	< 0.1	> 10,000	1,000	0.125	3,500	> 10,000	< 0.2	> 10,000	> 10,000	< 1	> 10,000
(±)-Methcathinone	> 10,000	< 0.1	> 10,000	***< 156***	***> 0.8***	***< 156***	> 10,000	< 0.2	> 10,000	> 10,000	< 1	> 10,000
(±)-Methylone	5,000	0.2	> 10,000	**< 156**	**> 0.8**	**< 156**	> 10,000	< 0.2	> 10,000	> 10,000	< 1	> 10,000
(±)-4-MEC	7,500	0.13	> 10,000	**< 156**	**> 0.8**	**< 156**	> 10,000	< 0.2	> 10,000	> 10,000	< 1	> 10,000
(±)-Flephedrone	> 10,000	< 0.1	> 10,000	**< 156**	**> 0.8**	**< 156**	> 10,000	< 0.2	> 10,000	> 10,000	< 1	> 10,000
(±)-Butylone	**156**	**6.4**	**900**	**< 156**	**> 0.8**	**< 156**	> 10,000	< 0.2	> 10,000	> 10,000	< 1	> 10,000
mCPP	> 10,000	< 0.1	> 10,000	> 10,000	< 0.0125	> 10,000	> 10,000	< 0.2	> 10,000	> 10,000	< 1	> 10,000
(±)-Methedrone	> 10,000	< 0.1	> 10,000	**< 156**	**> 0.8**	**< 156**	> 10,000	< 0.2	> 10,000	> 10,000	< 1	> 10,000
5-MeO-DiPT	> 10,000	< 0.1	> 10,000	5,000	0.025	> 10,000	> 10,000	< 0.2	> 10,000	> 10,000	< 1	> 10,000
(±)-DOB	> 10,000	< 0.1	> 10,000	> 10,000	< 0.0125	> 10,000	> 10,000	< 0.2	> 10,000	> 10,000	< 1	> 10,000
2C-B	> 10,000	< 0.1	> 10,000	> 10,000	< 0.0125	> 10,000	> 10,000	< 0.2	> 10,000	> 10,000	< 1	> 10,000
DMT	> 10,000	< 0.1	> 10,000	> 10,000	< 0.0125	> 10,000	> 10,000	< 0.2	> 10,000	> 10,000	< 1	> 10,000
BZP	> 10,000	< 0.1	> 10,000	> 10,000	< 0.0125	> 10,000	> 10,000	< 0.2	> 10,000	> 10,000	< 1	> 10,000
AMT	> 10,000	< 0.1	> 10,000	> 10,000	< 0.0125	> 10,000	> 10,000	< 0.2	> 10,000	> 10,000	< 1	> 10,000
2C-I	> 10,000	< 0.1	> 10,000	> 10,000	< 0.0125	> 10,000	> 10,000	< 0.2	> 10,000	> 10,000	< 1	> 10,000
2C-T-7	> 10,000	< 0.1	> 10,000	> 10,000	< 0.0125	> 10,000	> 10,000	< 0.2	> 10,000	> 10,000	< 1	> 10,000
TFMPP	> 10,000	< 0.1	> 10,000	> 10,000	< 0.0125	> 10,000	> 10,000	< 0.2	> 10,000	> 10,000	< 1	> 10,000
2C-T-4	> 10,000	< 0.1	> 10,000	> 10,000	< 0.0125	> 10,000	> 10,000	< 0.2	> 10,000	> 10,000	< 1	> 10,000

[a] Concentration of the drug that produces an absorbance reading equivalent to the 10 ng/mL cut-off of the targeted analyte.
[b] Concentration of the drug that produces an absorbance reading equivalent to the 1.25 ng/mL cut-off of the targeted analyte.
[c] Concentration of the drug that produces an absorbance reading equivalent to the 20 ng/mL cut-off of the targeted analyte.
[d] Concentration of the drug that produces an absorbance reading equivalent to the 100 ng/mL cut-off of the targeted analyte.
Analytes demonstrating high cross-reactivity are **highlighted and bolded**.
The target analytes for each reagent are ***italicized and bolded***.

Table 11: Summary of Cross-Reactivity Data for Designer Drug Stimulants in Commercial Immunoassays.

	Immunalysis		Neogen								Randox		Orasure			
Drug	Amp	Meth	Amp	AMP Specific	Amp Ultra	BZP	Ketamine	MPD	Meth/ MDMA	MPT	MDPV	Meph/ Mcath	PCP	Cotinine	Amp Specific	Meth
(±)-Amphetamine	T		T	T	T				T						T	
(±)-Methamphetamine		T	■		■					■						T
2C-E																
(±)-DOET																
(±)-DOM																
(±)-TMA																
(±)-MDA	■			■											■	■
(±)-MDEA		■	■		■				■							■
(±)-MDMA		■	■		■				T	■						■
(±)-Ethylamphetamine		■	■		■				■							■
(±)-MDPV											T					
(±)-Mephedrone												T				■
(±)-Cathinone																
(±)-Methcathinone												T				■
(±)-Methylone												■				■
(±)-4-MEC												■				■
(±)-Flephedrone												■				■
(±)-Butylone											■	■				■
mCPP			■		■											
(±)-Methedrone												■				■
5-MeO-DiPT																
(±)-DOB																
2C-B																
DMT																
BZP						T										
AMT	■		■	■											■	
2C-I																
2C-T-7																
TFMPP																
2C-T-4																

T = Target analyte

■ = Cross-reactive at concentrations <650 ng/mL

Amp - amphetamine; BZP - benzylpiperazine; Meph - mephedrone; Meth - methamphetamine; Mcath - methcathinone; MPD - methylphenidate; MPT - mephentermine

Table 12: Cross-Reactivity Data for EMIT Reagents.

	Syva			
	Amphetamines		Ecstasy	
Drug	C_{300}[a] (ng/mL)	Cross-Reactivity (%)	C_{300}[a] (ng/mL)	Cross-Reactivity (%)
(+)-Methamphetamine	***300***	***100***	> 10,000	< 3
(±)-Amphetamine	**< 2,000**	**> 15**	> 10,000	< 3
2C-E	> 10,000	< 3	> 10,000	< 3
(±)-DOET	> 10,000	< 3	> 10,000	< 3
(±)-DOM	> 10,000	< 3	> 10,000	< 3
(±)-TMA	> 10,000	< 3	> 10,000	< 3
(±)-MDA	**< 2,000**	**> 15**	**< 2,000**	**> 15**
(±)-MDEA	4,000	7.5	**< 2,000**	**> 15**
(±)-MDMA	4,000	7.5	***300***	***100***
(±)-Ethylamphetamine	**< 2,000**	**> 15**	4,000	7.5
(±)-MDPV	> 10,000	< 3	> 10,000	< 3
(±)-Mephedrone	> 10,000	< 3	> 10,000	< 3
(±)-Cathinone	> 10,000	< 3	> 10,000	< 3
(±)-Methcathinone	> 10,000	< 3	> 10,000	< 3
(±)-Methylone	> 10,000	< 3	> 10,000	< 3
(±)-4-MEC	> 10,000	< 3	> 10,000	< 3
(±)-Flephedrone	> 10,000	< 3	> 10,000	< 3
(±)-Butylone	> 10,000	< 3	4,000	7.5
mCPP	> 10,000	< 3	> 10,000	< 3
(±)-Methedrone	> 10,000	< 3	> 10,000	< 3
5-MeO-DiPT	> 10,000	< 3	> 10,000	< 3
(±)-DOB	3,000	10	> 10,000	< 3
2C-B	> 10,000	< 3	> 10,000	< 3
DMT	> 10,000	< 3	> 10,000	< 3
BZP	> 10,000	< 3	> 10,000	< 3
AMT	4,000	7.5	> 10,000	< 3
2C-I	> 10,000	< 3	> 10,000	< 3
2C-T-7	> 10,000	< 3	> 10,000	< 3
TFMPP	> 10,000	< 3	> 10,000	< 3
2C-T-4	> 10,000	< 3	> 10,000	< 3

[a] Concentration of the drug that produces an absorbance reading equivalent to the 300 ng/mL cut-off.
Analytes demonstrating high cross-reactivity are **highlighted and bolded**.
The target analytes for each reagent are ***italicized and bolded***.

Table 13: Results of Blind Spiked Sample Analysis by ELISA.

	Immunalysis		Neogen								Randox		Orasure			
Sample	**Amp**	**Meth**	**Amp**	**AMP Specific**	**Amp Ultra**	**BZP**	**Ketamine**	**MPD**	**Meth/ MDMA**	**MPT**	**MDPV**	**Meph/ Mcath**	**PCP**	**Cotinine**	**Amp Specific**	**Meth**
Sample 01	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-
Sample 02	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-
Sample 03	-	+	+	-	+	-	-	-	±	-	±	±	-	+	-	+
Sample 04	-	+	+	-	+	-	-	-	±	-	±	-	-	+	-	+
Sample 05	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Sample 06	-	+	+	-	+	-	-	-	+	-	-	+	-	+	-	+
Sample 07	-	+	+	-	+	-	-	-	+	-	-	+	-	+	-	+
Sample 08	-	+	+	-	+	-	-	-	+	±	±	-	-	+	-	+
Sample 09	-	+	+	-	+	±	-	-	+	±	-	-	-	+	-	+
Sample 10	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+
Sample 11	+	-	+	+	+	-	-	-	-	-	-	-	-	+	+	-
Sample 12	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	±
Sample 13	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Sample 14	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-	+
Sample 15	+	+	+	+	+	-	-	-	±	-	±	+	-	+	+	+
Sample 16	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Sample 17	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Sample 18	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Sample 19	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Sample 20	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Sample 21	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-
Sample 22	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-

Table 14: LC-QTOF Data for Blind Spiked Samples.

Sample	Match	Observed Mass	Target Mass	Difference (ppm)	Score
Sample 01	Methylone	207.0886	207.0895	-4.3	94.99
Sample 02	Methylone	207.0889	207.0895	-2.9	94.45
Sample 03	Ethylamphetamine	163.1352	163.1361	-5.48	94.77
Sample 04	Ethylamphetamine	163.1354	163.1361	-4.58	96.91
Sample 05	None				
Sample 06	Methedrone	193.1086	193.1103	-8.54	79.17
Sample 07	Methylone	207.0883	207.0895	-5.96	91.92
Sample 08	2C-I	307.006	307.0069	-2.96	97.08
Sample 09	None				
Sample 10	Methylone	207.0892	207.0895	-1.47	96.22
	Flephedrone	181.0899	181.0903	-1.88	99.24
Sample 11	mCPP	196.0759	196.0767	-4.04	95.37
Sample 12	Methylone	207.0883	207.0895	-5.93	91.66
	5-MeO-DiPT	274.2034	274.2045	-4.18	93.90
Sample 13	2C-I	307.0057	307.0069	-4.05	94.85
	2C-T-7	255.128	255.1293	-5.16	92.26
Sample 14	Mephedrone	177.1146	177.1154	-4.23	96.86
	MDPV	275.1508	275.1521	-4.75	93.07
Sample 15	AMT	174.1145	174.1157	-6.62	80.41
	Butylone	221.1037	221.1052	-6.95	88.85
	Ethylamphetamine	163.1353	163.1361	-5.07	94.30
Sample 16	None				
Sample 17	2C-I	307.0078	307.0069	2.78	97.21
Sample 18	2C-I	307.0057	307.0069	-3.86	95.05
Sample 19	2C-I	307.0059	307.0069	-3.35	96.30
Sample 20	Flephedrone	181.0899	181.0903	-2.41	98.28
Sample 21	Flephedrone	181.0892	181.0903	-6.23	92.78
Sample 22	Flephedrone	181.0893	181.0903	-5.7	94.01

Table 115: Screening of PBSO Case Samples by ELISA

	Immunalysis		Neogen								Randox		OraSure			
Sample	**Amp**	**Meth**	**Amp**	**AMP Specific**	**Amp Ultra**	**BZP**	**Ketamine**	**MPD**	**Meth/ MDMA**	**MPT**	**MDPV**	**Meph/ Mcath**	**PCP**	**Cotinine**	**Amp Specific**	**Meth**
07-001	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11-001	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11-002	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
12-001	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
12-002	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-

Table 126: Screening of PBSO Case Samples by LC-QTOF

Sample	Library Matches
7-001	diphenhydramine, methadone, EDDP (methadone metabolite), alprazolam
11-001	caffeine
11-002	benzoylecgonine, cyclobenzaprine, 3-hydroxycotinine, alprazolam
12-001	benzoylecgonine, 3-hydroxycotinine, alprazolam
12-002	3-hydroxycotinine, cotinine, citalopram, norcitalopram, DMAA

COMPREHENSIVE FORENSIC TOXICOLOGICAL ANALYSIS OF DESIGNER DRUGS 2011-DN-BX-K559

Anthony P. DeCaprio, W. Lee Hearn and Madeleine J. Swortwood.

DRAFT FINAL REPORT
RESPONSE TO REVIEWER COMMENTS

REVIEWER #1:

Recommended Revisions:
a. ***Abstract -*** *Change "while manufacturers have been staying one step ahead of the law with constantly evolving modifications to structures." to "while manufacturers have been staying one step ahead of the law with constantly evolving modifications to* ***drug molecular*** *structures."*

RESPONSE: We agree with the comment and thank the Reviewer for the recommendation. This change has been reflected in the text.

b. ***Executive Summary -*** *the sentence "The major goal of the present project was to evaluate the performance of commercially available screening immunoassays for detecting a wide range of designer drugs." found in the third paragraph, is repeated again word for word in the fifth paragraph. Since statement has already been made, it is redundant to repeat it again.*

RESPONSE: This was a typo has been deleted from the fifth paragraph.

c. ***Table 4: Composition of Blind Spiked Samples for Task 2 Screening. –*** *the drug "methylone" appears to be listed as "methy lone" in four rows. (extra space between "methy" and "lone"). This might just be from the copy that the reviewer was given.*

RESPONSE: We believe this must be an issue with the original copy as our current version does not appear that way.

RESPONSE TO REVIEWER #2:

Recommended Revisions:
Confirmation of the identification of spiked drugs by mass spectrometry adds little to the results of the project and can be deleted to improve the clarity and conciseness of the report.

RESPONSE: We understand the Reviewer's point. However, the MS work was performed as confirmation for the analyst, as the results of the spiking study were blind until after completion of this portion of the research. Since the draft report was prepared, we have added five authentic forensic specimens to the study and the MS data are necessary to confirm the immunoassay results, as the specimens were submitted to us without any knowledge of the case or the submitting laboratory's initial findings. The report has therefore been updated with the results of the cases that were analyzed by ELISA, LC-QQQ-MS, and LC-QTOF-MS.