

# Matrix Effect and Cross-Reactivity of Select Amphetamine-Type Substances, Designer Analogues, and Putrefactive Amines using the Bio-Quant Direct ELISA Presumptive Assays for Amphetamine and Methamphetamine

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## Abstract

The aim of this study was to evaluate the Bio-Quant Direct ELISA assays for amphetamine and methamphetamine in the routine presumptive screening of biological fluids. Standard concentration curves of the target analytes were assayed to assess sensitivity, and known concentrations of common amphetamine-type substances (ephedrine, pseudoephedrine, phentermine), designer analogues (MDA, MDMA, MDEA, MBDB, PMA, 4-MTA, 2CB), and putrefactive amines (phenylethylamine, putrescine, tryptamine, tyramine) were analyzed to determine cross-reactivity. Results of the standard curve studies show the capacity of both Direct ELISA kits to confidently detect down to 3 ng/mL interday (PBS matrix; CVs 6.3–15.5%). Cross-reactivity relative to that of 50 ng/mL preparations of the target compounds demonstrated that the Direct ELISA kit for amphetamine also detected MDA (282%), PMA (265%), 4-MTA (280%), and phentermine (61%), and the Direct ELISA for methamphetamine also assayed positive for MDMA (73%), MDEA (18%), pseudoephedrine (19%), MBDB (8%), and ephedrine (9%). Matrix studies demonstrated that both ELISA kits could be applied to screening of blood, urine, and saliva to a concentration of 6 ng/mL or lower. In conclusion, the Bio-Quant Direct ELISA kits for amphetamine and methamphetamine are fast and accurate and have demonstrated themselves to be useful tools in routine toxicological testing.

## Introduction

Immunoassays are antibody-based analytical tests that identify and measure amounts of a chemical substance and are, in forensic toxicological application, typically used as presumptive

screening tools for drugs of abuse. Many different immunoassays are commercially available for toxicological analysis, and they generally differ in the compounds or drug-conjugates used for competitive binding and the method of detection (e.g. fluorescence or absorbance). Several references are available discussing the analysis of amphetamine-type substances and other drugs of abuse using immunoassay techniques such as radioimmunoassay (RIA), enzyme-multiplied immunoassay (EMIT<sup>®</sup>), fluorescence polarization immunoassay (FPIA), and cloned enzyme donor immunoassay (CEDIA<sup>®</sup>) (1–11). The focus of this study was to examine the applicability of enzyme-linked immunosorbent assay (ELISA) for the screening of forensic biological specimens while considering practical aspects such as interference from putrefactive amines and the cross-reactivity of analogues.

ELISA assays for drugs of abuse are marketed by several companies, and in the case of this study, the Bio-Quant Direct ELISA assays for amphetamine and for methamphetamine were selected (Bio-Quant, San Diego, CA). In typical ELISA systems for drugs of abuse screening, the supplied 96-well ELISA plate is coated with drug-specific antibody (e.g., anti-amphetamine, anti-methamphetamine). In solution, the enzyme-labeled drug conjugate (drug-horseradish peroxidase) converts a substrate (tetramethylbenzidine) to a colored product ( $\lambda_{\max} = 450 \text{ nm}$ ); the presence of free drug competitively restricts this activity resulting in an absorbance reading inversely proportional to the concentration of drug in the sample (12). The benefits of ELISA are numerous and include rapidity of procedural and analysis times, excellent sensitivity (0.01–1.0  $\mu\text{g/mL}$ ), antibody-based specificity, commercial availability, and wide acceptability in the analytical community (13).

Proprietary ELISA assays specific for amphetamine and methamphetamine have been evaluated for sensitivity and specificity in recent years, and have examined numerous amphetamine-type substances in matrices such as plasma, blood,

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urine, and oral fluid (14–18). For the evaluation of the specificity of the Bio-Quant Direct ELISAs for amphetamine and methamphetamine, this study has included amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyethamphetamine (MDEA), *p*-methoxyamphetamine (PMA), 4-methylthioamphetamine (4-MTA), 4-bromo-2,5-dimethoxyphenylethylamine (2CB), *N*-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine (MBDB), ephedrine, pseudoephedrine, and phentermine. Prior studies have demonstrated varying degrees of cross-reactivity for MDA and MDMA (14–17), MDEA (15,16), PMA and MBDB (15), ephedrine (14–16,18), pseudoephedrine (14,16), and phentermine (14). This study confirms the behavior of these compounds and adds two other designer analogues (4-MTA and 2CB) to create a consolidated reference of this class of drugs as applied to the Bio-Quant Direct ELISA systems.

In addition, cross-reactivity of the putrefactive amines phenylethylamine, putrescine, tryptamine, and tyramine with the Bio-Quant Direct ELISA was examined, an understanding of which would allow further confidence in the application of these ELISA assays to the routine toxicological testing of post-mortem blood, especially in cases when decomposition is apparent. Several papers over the last few decades have discussed the identification of putrefactive, or biogenic, amines (19–23), and others have addressed the potential of the putrefactives to interfere with antibody-based immunoassays such as EMIT (24–28). In addition, the potential of ELISA cross-reactivity with putrefactive amines has been directly examined with the inclusion of phenylethylamine (14) and tyramine (16) in specificity studies utilizing other commercially available kits.

Therefore, the goals of this study were threefold: 1. to assess the sensitivity and precision of the amphetamine and methamphetamine Bio-Quant Direct ELISA kits and hence their application to practical toxicology; 2. to examine the specificity of the amphetamine and methamphetamine Bio-Quant Direct ELISA assays by examining the cross-reactivity of several common amphetamine-type substances, designer analogues, and naturally occurring putrefactive amines; and 3. to assess matrix influence.

## Experimental

### Samples and reagents

Reference standards (hydrochloride salts) of amphetamine, methamphetamine, MDA, MDMA, MDEA, PMA, 4-MTA, 2CB, MBDB, ephedrine, pseudoephedrine, phentermine, phenylethylamine, putrescine, tryptamine, and tyramine were obtained from the reference material division of the National Measurement Institute (Sydney, Australia), and working stock solutions were prepared at concentrations of 10 µg/mL in phosphate-buffered saline (PBS, pH 7.4). Donated drug-free blood was pre-screened in-house to confirm the absence of medications and illicit drugs. Synthetic urine positive (50 ng/mL) and negative controls were supplied with each ELISA kit. Drug-free saliva was collected interday (single-source) and

centrifuged at 3000 rpm for 3–5 min. PBS (pH 7.4) was prepared from dissolution of tablets (AMRESCO, Solon, OH) in deionized water.

### Apparatus

Assay plate wash procedures were performed using deionized water and a plate washer apparatus (BioTek Instruments, Winooski, VT). Absorbance readings were determined at dual wavelength (450 nm, 650 nm reference) using the ELx800 plate reader (BioTek Instruments).

## Methods

### Standard curve preparation

To evaluate the sensitivity and range of linearity of the Bio-Quant Direct ELISA kits for amphetamine and methamphetamine, standards curves of both individual target compounds were prepared in PBS (pH 7.4). In addition to a blank negative control in PBS, standard concentrations were prepared from serial dilution of a 50 ng/mL preparation, giving final concentrations of 50, 25, 13, 6, 3, and 0 ng/mL for assay.

### Cross-reactivity/specificity

For assessment of specificity, standard solutions of common amphetamine-type substances (amphetamine, methamphetamine, ephedrine, pseudoephedrine, phentermine), designer analogues (MDA, MDMA, MDEA, PMA, 4-MTA, 2CB, MBDB), and putrefactive amines (phenylethylamine, putrescine, tryptamine, tyramine) were prepared at a concentration of 50 ng/mL in PBS (pH 7.4). The concentration of 50 ng/mL was chosen because it is well above the detection limits of the target amphetamine and/or methamphetamine and near what could be utilized as a cut-off value in routine analysis. PBS (pH 7.4) was chosen as the control matrix for cross-reactivity study to eliminate contributions from interferences present in the biological specimens.

### Matrix effect

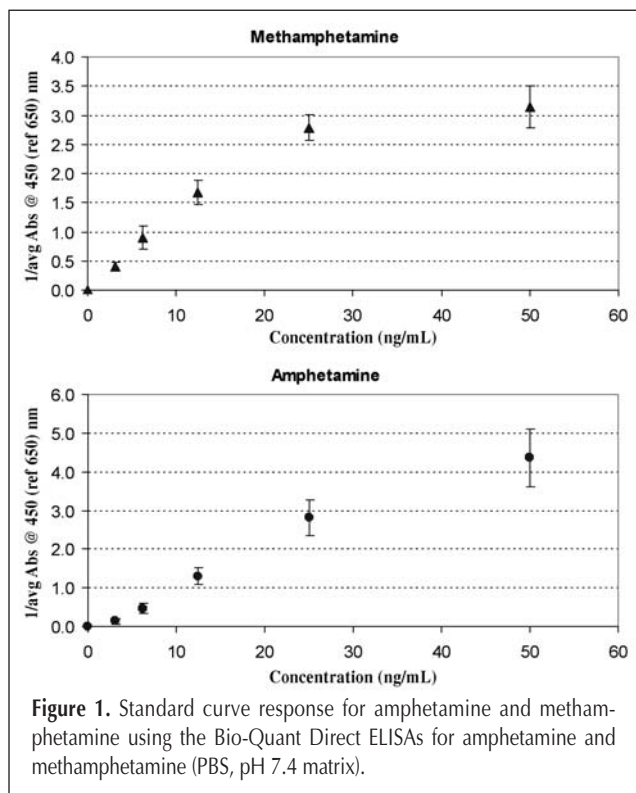
In each of the interday analyses, samples containing 50 ng/mL amphetamine or methamphetamine were prepared in whole blood and saliva matrices for comparison against the supplied positive urine control and the PBS (pH 7.4) preparation. In addition, concentrations of either amphetamine or methamphetamine (0, 6, 13, and 50 ng/mL) were prepared by serial dilution in saliva, whole blood, dilute whole blood (1:4, v/v, with PBS, pH 7.4), and in the supplied urine control(s) to be used for study of matrix influence on absorbance.

### ELISA procedure

The Bio-Quant Direct ELISA is a competitive antibody-based enzyme immunoassay designed to detect drugs of abuse in forensic applications. The assays were performed according to the instructions of the manufacturer, and each assay consisted of target standard preparations, a positive urine control, and negative controls. All standards and controls were analyzed as replicates in successive wells.

After pipetting 10 µL of standards, controls, and sample

preparations to the appropriate wells of the 96-well plate, 100  $\mu$ L of drug-enzyme conjugate (drug-horseradish peroxidase) was added to each well, mixed, and incubated at room tem-



**Figure 1.** Standard curve response for amphetamine and methamphetamine using the Bio-Quant Direct ELISAs for amphetamine and methamphetamine (PBS, pH 7.4 matrix).

**Table I. Relative Cross-Reactivity of Common Amphetamine-Type Substances, Designer Analogues, and Putrefactive Amines using the Bio-Quant Direct ELISA Assays for Methamphetamine (MA) and Amphetamine (AM)\***

Compound (50 ng/mL)	Bio-Quant Methamphetamine Direct ELISA (n = 12)		Bio-Quant Amphetamine Direct ELISA (n = 14)	
	Relative cross-reactivity (%)	Concentration equivalent to 50 ng/mL MA	Relative cross-reactivity (%)	Concentration equivalent to 50 ng/mL AM
Amphetamine	2	2080	N/A	N/A
Methamphetamine	N/A	N/A	–	–
MDA	–	–	282	18
MDMA	73	69	–	–
MDEA	18	284	–	–
MBDB	8	627	–	–
Ephedrine	9	561	–	–
Pseudoephedrine	19	267	–	–
PMA	4	1247	265	19
4-MTA	5	941	280	18
2CB	–	–	–	–
Phentermine	–	–	61	81
Phenylethylamine	–	–	2	2226
Putrescine	–	–	–	–
Tryptamine	–	–	–	–
Tyramine	–	–	–	–

\* All substance concentrations were assayed at 50 ng/mL and compared versus the target analyte of amphetamine or methamphetamine.

perature for 60 min. The plate was then washed repeatedly with deionized water utilizing a plate washer (Bio-Tek Instruments), and 100  $\mu$ L of substrate (3,3',5,5'-tetramethylbenzidine and peroxide in buffer) was added and incubated for 30 min at room temperature. Finally, 100  $\mu$ L of stop solution (1N hydrochloric acid) was added, and the absorbance of each well at dual wavelength (450 and 650 nm) was determined using the ELx800 plate reader. A sample is determined to be positive if the average absorbance is equal to or less than that of the laboratory positive reference standard.

## Results

In the Bio-Quant Direct ELISAs for methamphetamine and amphetamine, the standard curves exhibited a non-linear response distribution characteristic of ELISA immunoassays (29) (Figure 1). In the PBS matrix, the absorbance of a positive reading for either methamphetamine or amphetamine was distinguishable from the 0 ng/mL negative controls interassay throughout the concentration range tested (methamphetamine:  $n = 12$  at each concentration, CVs < 16%; amphetamine:  $n = 14$  at each concentration, CVs < 18%). The results from these standard curves are largely consistent with the supplied quality assurance statements for LOD (1 ng/mL methamphetamine/amphetamine), as well as with typical control standard absorbance values obtained in manufacturer precision studies. It is demonstrated that the Direct ELISA systems are applicable

for the sensitive determination of these amphetamine-type substances as either target compound or principle metabolite.

To determine cross-reactivity, responses from the various amphetamine-type substances, designer analogues, and putrefactive amines were statistically compared and reported relative to that of the 50 ng/mL target analyte standard. Favorable repeatability was observed for cross-reactivity values obtained in the methamphetamine system over multiple assays ( $n = 12$ , CVs < 19%) (Table I, Figure 2). It was observed that several of the substances tested reacted, with the most significant being MDMA (73%), MDEA (18%), pseudoephedrine (19%), MBDB (8%), and ephedrine (9%). In comparison with the Bio-Quant Direct ELISA for methamphetamine, the amphetamine ELISA offered a higher degree of specificity. Only MDA, PMA, 4-MTA, and phentermine exhibited cross-reactivity to a significant degree: 282%, 265%, 280%, and 61%, respectively (Table I, Figure 2). Interassay reproducibility was again favorable ( $n = 14$ , CVs < 18%, with the exception of PMA at 26%).

To assess the potential matrix influence on response, each assay also included samples prepared in multiple lots of saliva (single-source), whole blood, and the supplied syn-

thetic urine control at concentrations of 50 ng/mL ( $n = 12$  total replicates for methamphetamine;  $n = 14$  for amphetamine). In the Direct ELISA for methamphetamine, little variation was observed for the saliva, PBS, and urine matrices (CVs 11%), whereas a greater degree of variation was observed for the whole blood matrix (CV 32%). Figure 3 illustrates that in these trials, average absorbance at 450 nm was lowest for the saliva (0.22) and PBS matrices (0.27), followed by that of the whole blood sample (0.41) and synthetic urine control (0.51). In the Direct ELISA for amphetamine, the whole blood sample exhibited the higher absorbance interday (0.35, CV 9%). The urine control gave the lowest average response in this system (0.14, CV 18%), and saliva and PBS exhibited reproducible re-

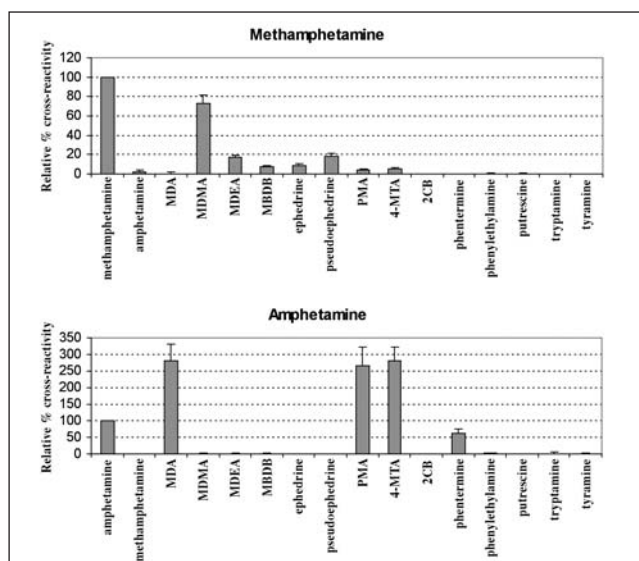
sponse at 0.17 (CV 12%) and 0.21 (CV 18%), respectively. Additionally, the concentration study presented in Figure 4 reinforced these trends.

### Discussion

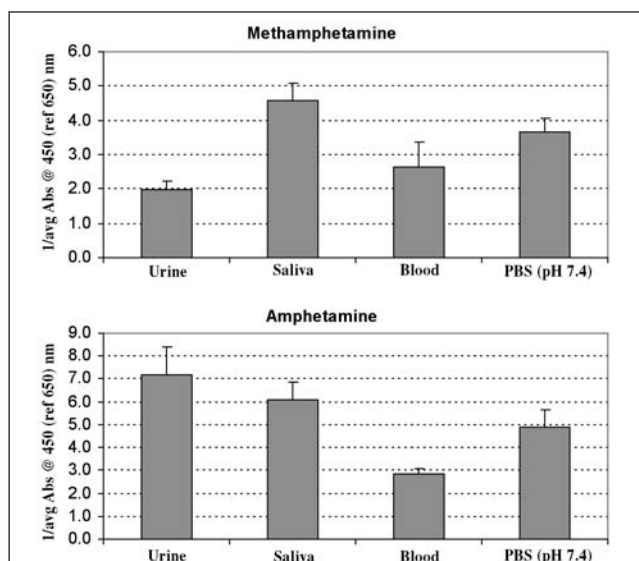
With regards to the sensitivity of the assays for the target methamphetamine or amphetamine, the quality assurance statements provided state a capability to effectively detect a concentration of 1 ng/mL. The results obtained from these standard curve studies support this limit of detection capacity (Figure 1) with acceptable precision. The levels of positive detection exhibited in these trials demonstrate both Direct ELISA systems to be acceptable for routine analysis, and reinforces the advantages of ELISA in terms of sensitivity. This is true for both the non-biological PBS matrix and the biological matrices investigated in this study (Figure 4).

Assessment of relative cross-reactivity revealed that several of the substances tested reacted to the methamphetamine system, the most significant again being MDMA, MDEA, pseudoephedrine, MBDB, and ephedrine (Table I, Figure 2). In illicit methamphetamine screening, cross-reactivity of pseudoephedrine and ephedrine is of concern because of the potential for reporting false positives in unconfirmed samples. However, the cross-reactivity of designer analogues can be of significant advantage by highlighting their presence.

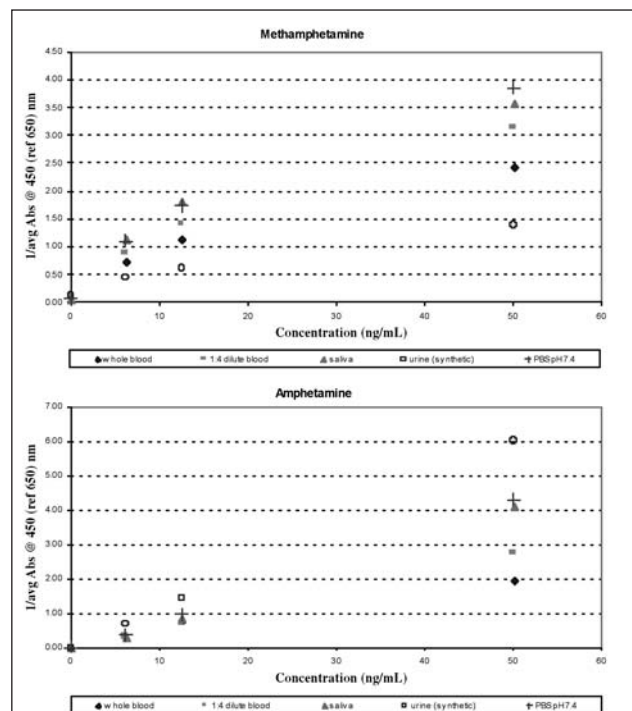
The direct ELISA for amphetamine also demonstrated significant cross-reactivity with other amines (Table I, Figure 2). In this case, MDA and the more novel PMA and 4-MTA ana-



**Figure 2.** Relative cross-reactivity of common amphetamine-type substances, designer analogues, and putrefactive amines versus target amphetamine or methamphetamine (all substances assayed at a concentration of 50 ng/mL; PBS, pH 7.4 matrix).



**Figure 3.** Comparison of the influence of matrix on the absorbance response of the Bio-Quant Direct ELISA assays for amphetamine or methamphetamine (assay concentration = 50 ng/mL).



**Figure 4.** Comparison of matrix influence on the absorbance response of the Bio-Quant Direct ELISA assays for amphetamine or methamphetamine; concentration curves prepared in whole blood, dilute blood (1:4, v/v, with PBS, pH 7.4), saliva, urine (synthetic), and PBS (pH 7.4).

logues exhibited reactivity much higher than amphetamine, and phentermine was also detectable. Minor levels of cross-reactivity have been reported for a number of structurally similar drugs of the amphetamine class, and the aforementioned published ELISA studies (14–18) repeatedly indicate the potential of such substances to cross-react with methamphetamine and amphetamine antibody-based immunoassays. This is also supported by the cross-reactivity data provided by the manufacturer. The results seen in this study therefore further support the necessity to implement an appropriate in-house cut-off value to minimize the risk of presumptive false positives, while offering the ability to simultaneously screen for several novel amphetamine-type substances and designer analogues. In addition, concurrent use of the two assays, Direct ELISA for amphetamine and methamphetamine, allows for a practical means to determine the presence of both parent drug and primary metabolite (e.g., methamphetamine and amphetamine, MDMA and MDA) in a range of biological fluid matrices.

As the influence of matrix substituents is a significant consideration in any analysis, it is generally required that method validation be performed incorporating the matrix which is routinely tested. In these studies, a PBS matrix was selected to eliminate matrix contributions while evaluating the cross-reactivity of naturally occurring amines relating to biological and/or postmortem specimens. However, it was observed in the other matrices tested (saliva, whole blood, and urine) that there can be clear differences in the absorbance values obtained (Figures 3 and 4). Also of significance is that the trends in matrix influence and response are not necessarily consistent between the methamphetamine and amphetamine immunoassays. In the former, average absorbance was lowest for the saliva and PBS matrices, followed by that of the whole blood sample and synthetic urine control. In the amphetamine system, the urine control gave the lowest average response, then saliva and PBS, with the whole blood specimen exhibiting the highest absorbance. The concentrations of the supplied controls were not verified by other means [e.g., gas chromatography–mass spectrometry (GC–MS)] and may be contributing to the difference in response between these and the in-house preparations. Another factor to consider is that non-diluted blood can result in stronger response. The manufacturer reports that hemoglobin can bind non-specifically to the well and subsequently react with 3,3',5,5'-tetramethylbenzidine to cause an increase in background color. Irrespective of the differences observed between the different matrices and both immunoassay systems, these results do demonstrate the applicability of the Direct ELISA systems for use with the various biological fluids, and reinforce the need for validation efforts to include each matrix of interest. Matrix effects at lower concentrations than the aforementioned 50 ng/mL were also investigated by including levels of 13 and 6 ng/mL in-matrix preparations by serial dilution (Figure 4). The concentration curves in Figure 4 support the pattern of matrix influence seen in both the Direct ELISA for amphetamine and methamphetamine (intraday replicate deviation from < 1% to 13%), and also display several other interesting outcomes.

In most instances, the analysis of PBS resembled that of saliva. Testing of diluted blood (1:4, v/v, with PBS, pH 7.4)

confirmed the manufacturer recommendation that this specimen type may reduce background noise, as the average absorbance improved in both assays in comparison to whole blood. It should be noted, however, that dilution can also by definition reduce sensitivity. The Direct ELISA for amphetamine also exhibited a linear response in the range studied and most matrices (whole blood being the exception) had correlation coefficients greater than 0.99. Most significantly, again in support of the applicability of the Direct ELISA for a range of biological matrices, is that a concentration of 6 ng/mL is detectable in all matrices; this concentration is well below a likely cut-off value in routine presumptive screening and reflects a subtherapeutic level of toxicological relevance.

In the standard curve, specificity, and matrix effect studies, a considerable degree of interassay variability was observed. Standard deviation and CV were significant in some cases (e.g. CV > 20%), which may be attributable to different ELISA kit lots, stability of the working drug standard solutions, or analytical technique. Variation in absorbance reading due to the spectrophotometric plate reader was discounted, as a certified filter plate was tested prior to each analysis being performed. It should be noted, however, that immunoassays have been recognized as inherently imprecise, and that stipulations regarding their validation are recommended to be less restrictive (e.g., precision acceptance > 20% CV) (29). In any case, the assays performed well as a non-quantitative presumptive assay in that they tested positive for appropriate concentrations of the target compounds in every assay performed.

These results show the potential of the Bio-Quant Direct ELISA assays to be rapid and accurate for the routine toxicological screening for amphetamine-type substances and designer analogues. Results of standard curves exhibit suitable subtoxic detection limits, and observed drug class-specific relative cross-reactivity indicates use of the kits may detect other illicit amines in addition to the target compounds. In the screening of post-mortem blood, the application of both the amphetamine kit and the methamphetamine kit could prove to be a useful analytical tool, particularly in the detection of parent drug and metabolites, although it is unlikely that false-positives would occur due to extraordinary concentrations of putrefactive bases. To summarize, the benefits of ELISA in toxicological screening of various matrices for amphetamines have been demonstrated here. Additionally, the false-positive rate due to the licit or incidental compounds investigated in this study is minimal. Subsequent confirmation by a quantitative method such as GC–MS or liquid chromatography–MS is still always recommended.

## Conclusions

These data indicate that the Bio-Quant Direct ELISA assays for the detection of amphetamine and methamphetamine comprise a rapid and reliable technique for the presumptive screening of forensic samples. Standard curves have demonstrated subtherapeutic detection limits of toxicological relevance (< 3 ng/mL), and observed relative cross-reactivity has confirmed the capability of detecting other illicit or designer

amines in addition to the target compounds. This may be of significant advantage to the working laboratory. Study of matrix effect demonstrated the applicability of the ELISA kits to be effective for the screening of blood, urine, and saliva to concentrations at or below 6 ng/mL. In addition, it has been demonstrated that the Bio-Quant Direct ELISA kits are unlikely to be susceptible to false-positives due to putrefactive amines, thereby affirming confidence in their use for screening postmortem/decomposed blood specimens. In conclusion, the Bio-Quant Direct ELISA kits for amphetamine and methamphetamine are fast and accurate and have demonstrated themselves to be useful tools for immunoassay screening of forensic biological specimens.

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