

RESEARCH ARTICLE

Development of an Optimized Extraction Method to Recover Drug Material From Used Test Strips for Comprehensive Drug Checking

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ABSTRACT

Drug-checking programs use point-of-need testing (e.g., test strips) and laboratory-based analysis to rapidly identify emerging drug threats, but each has limitations. Test strips are quick but compound or class specific, whereas laboratory testing can identify more compounds but have lengthy turnaround times. To address these limitations, it was proposed that compounds could be extracted from used test strips for additional analyses allowing for rapid on-site information followed by comprehensive laboratory results. The method development process involved four parts: determining the optimal extraction approach, assessing the feasibility of performing direct analysis in real-time mass spectrometry (DART-MS) analysis on extracts, determining the limits of detection (LODs) for a range of analytes, and evaluating the method using used test strips submitted by harm reduction sites. The optimized method consisted of extracting analytes of interest from a cut test strip using 0.5-mL methanol while vortexing for 10 s. DART-MS successfully identified the compounds of interests, and the test strip chemical background was identified. LODs were found to be as low as a mass fraction of 0.005 in a mixture. For the samples submitted by harm reduction sites, concordance between extracts and test strip results was 96%, and the agreement in compound identification between used test strip extracts and authentic drug collection samples was approximately 80% regardless of test strip type and preparation. This work shows that additional analyses of extracted test strips can provide a low-barrier way for high-quality testing that can be used to increase data on the drug landscape.

1 | Introduction

The United States is experiencing a drug epidemic with fatal overdoses increasing by 58% over the past 5 years [1]. As part of the response to this epidemic, public health and harm reduction agencies have established drug-checking programs to better

inform people who use drugs (PWUD) what is in the drug supply and provide guidance and supplies for safer use. Informing PWUD about dangers in the drug supply requires analysis of drug product and/or paraphernalia to identify known and novel substances. This testing can be done either on-site, through point-of-need testing, or in a laboratory setting.

Certain commercial products are identified to adequately specify the procedure; this does not imply endorsement or recommendation by NIST nor does imply that such products are necessarily the best available for the purpose.

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Common analysis techniques for point-of-need drug checking include Fourier transform infrared spectroscopy (FTIR) [2] and lateral flow immunoassays (herein referred to as test strips) [3, 4]. Test strips are paper-based antibody tests that can detect one or more specific analyte within minutes. These tests, initially developed for detecting drugs in biological matrices, are easy to use, low-cost, highly sensitive, and can provide information to PWUD before consumption [5]. Recent studies have shown the effectiveness of these test strips for drug-checking applications but have also identified limitations including cross-reactivity and compound/class specificity [6, 7]. Most test strips can only detect one compound or class of compounds, making the information obtained from the tests limited in utility (e.g., xylazine is or is not present in a sample). The outcome of this limitation is the need to use multiple types of test strips, but even this approach does not provide complete insight into the sample, as there are not tests available for all compounds of interest. To obtain a complete picture of what is contained in a drug sample, extensive laboratory-based testing is needed.

Laboratory-based drug checking involves agencies submitting drug product, paraphernalia, or trace residue samples collected in the field to a laboratory for comprehensive analysis [8]—commonly using gas chromatography–mass spectrometry (GC–MS) [9, 10], liquid chromatography–mass spectrometry (LC–MS) [9, 10], and/or direct analysis in real-time mass spectrometry (DART-MS) [11]. Depending on the legal landscape of a particular jurisdiction, obtaining drug product or trace residue samples can present numerous challenges. One challenge is the legal restrictions on possessing drug material/paraphernalia both by PWUD and by drug-checking programs [12]. Some states allow for the possession of paraphernalia for the strict purpose of conducting drug checking, but in many states, this is not the case. There are also exposure risks associated with collecting drug product samples, which could impact the willingness of personnel to collect samples.

One alternative to overcome these challenges is to establish a way to obtain a comprehensive chemical profile of a drug sample from used test strips. Because many agencies already either conduct drug checking with test strips on-site or provide test strips to PWUD for personal testing, being able to utilize them for comprehensive testing eliminates the need to further handle drug product or to transport drug paraphernalia. It also offers a mechanism that provides PWUD with immediate information about some of the dangers of their sample while enabling the ability to provide retrospective information for broader education or monitoring purposes without requiring any additional drug product. The goal of this project was to determine if comprehensive drug profiles could be obtained from used test strips through extraction and analysis by mass spectrometry. In this study, we first optimized a method for the extraction of drug residues from used test strips that enabled further analysis by DART-MS. The performance of the extraction method was then characterized to identify limitations. Finally, the protocol was evaluated against traditional laboratory-based drug-checking approaches (drug product testing and trace residue collection) to understand the strengths and, more importantly, the limitations of this approach. The method described here provides a way for laboratories to be able to analyze used test strips and lower the

barrier for obtaining samples necessary for understanding the illicit drug market.

2 | Materials and Methods

This study was broken into four parts: (1) optimizing an extraction approach, (2) determining the feasibility of completing DART-MS analysis on used test strip extracts, (3) establishing approximate limits of detection (LOD) from test strip extractions, and (4) evaluating the extraction protocol using authentic samples.

2.1 | Chemicals and Materials

Fentanyl and xylazine test strips (BTNX Inc., Pickering, Ontario, Canada), with detection thresholds of 200 and 1000 ng/mL, respectively, were utilized to create “used” test strips in-house. Extraction solvents included acetonitrile, methanol, and water purchased from Sigma-Aldrich (St. Louis, Missouri, United States).

The first two parts of this study used a dual-component stock solution containing cocaine (Sigma-Aldrich) and xylazine (Sigma-Aldrich) in water at concentrations of 580 and 890 µg/mL, respectively.

For the LOD study, six-component solutions containing cocaine, fentanyl, heroin, isotonitazene, methamphetamine, and xylazine HCl (Cayman Chemical, Ann Arbor, Michigan, United States) were prepared at concentrations of 150, 100, 50, 10, 5, and 1 µg/mL in water. A second single-component solution set containing carfentanil (Cayman Chemical) in water at 1, 10, and 50 µg/mL was also created.

For quantitative analysis by liquid chromatography tandem mass spectrometry (LC–MS/MS), a 1-µg/mL internal standard solution was made by combining 0.5 mL of 1-mg/mL cocaine-*d*₃ in methanol (Cayman Chemical) and 1-mg/mL xylazine-*d*₆ in methanol (Cayman Chemical) and diluting to a final volume of 500 mL in methanol.

2.2 | Test Strip Preparation for Extraction Optimization and DART-MS Feasibility

To ensure that a known and consistent amount of material was adsorbed by the test strips, 0.05 mL of the cocaine/xylazine dual-component solution was pipetted onto the adsorbent pad of individual xylazine or fentanyl test strips and allowed to dry overnight. This approach resulted in each test strip being doped with approximately 29 µg of cocaine and 44.5 µg of xylazine HCl.

2.3 | Test Strip Extraction

The majority of this study used one of the two test strip extraction approaches—an initial, non-optimized approach (Procedure 1) and the optimized approach (Procedure 2). In Procedure 1, test strip extracts were prepared by cutting the test strip at Cut

Location 1, shown in Figure 1, and placing it into a 2-mL glass vial. Various solvents (1 mL) were added to the vial and capped. The vial was then vortexed for 10 s at 1047 rad/s (10,000 rpm), after which the test strip was removed to prevent reabsorption. In Procedure 2, test strip extracts were prepared by cutting the test strip at Cut Location 1, shown in Figure 1, and placing into a 2-mL glass vial. Methanol (0.5 mL) was added to the vial. The vial was then capped and vortexed for 10 s at 1047 rad/s (10,000 rpm), after which the test strip was removed to prevent reabsorption. Instances where these approaches were not followed are explicitly discussed in the Results and Discussion section. Cut Location 2 was used for a solvent follow-up study.

2.4 | Optimization of Test Strip Extraction

The development of an optimized extraction approach was broken down into three subparts focused on (1) solvent selection, (2) optimization using a design of experiments (DOE) approach, and (3) refinement of solvent volume. Extracts were quantified by LC-MS/MS to measure the solution concentration and percent recovery of cocaine and xylazine.

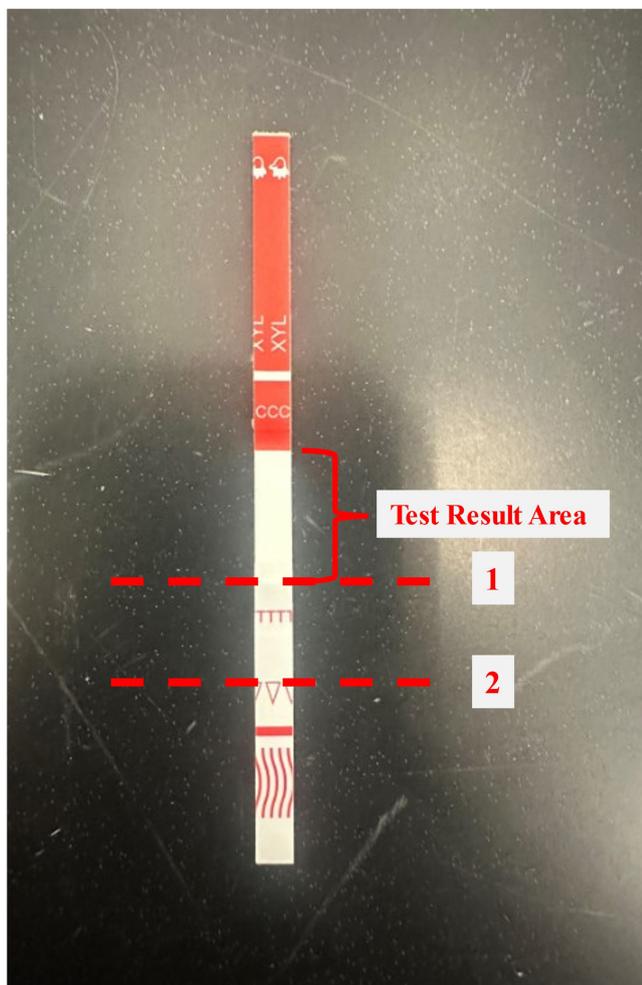


FIGURE 1 | Example of xylazine test strip showing Cut Locations 1 and 2 and test result area.

Solvent selection was completed by doping xylazine test strips with cocaine and xylazine (outlined previously) and extracting them using Procedure 1 with methanol, acetonitrile, water, or a 50:50 methanol:water mix. The average concentration of five replicate measurements for each solvent was used to narrow the list of solvent options for future analyses.

Following solvent selection, a DOE approach was used to understand the effects of solvent, solvent volume, test strip preparation, vortexing, extraction time, and test strip type. Two levels were chosen for each of the six parameters, and the 32 parameter sets were analyzed in triplicate (listed in Table S3). The average percent recoveries of cocaine and xylazine for each variable across all 32 parameter sets (shown in Figure 2) were used to inform the optimal extraction approach.

Finally, based on the results from the DOE, additional solvent volumes were compared to optimize the extraction volume. Incorporation of glass inserts was also studied to increase the contact of the solvent and test strip when using small volumes. Solvent volumes of 0.2 mL (with and without an insert), 0.5 mL, and 1 mL were studied using Procedure 1 and methanol as the solvent. Duplicate samples were run, and the percent recovery of cocaine and xylazine was used to inform the optimal solvent volume.

2.5 | DART-MS Feasibility

Determining the feasibility of completing DART-MS analysis on used test strip extracts was broken into two parts focused on (1) successful detection of compounds of interest in DOE samples and (2) understanding the chemical background from test strips. All DOE samples previously analyzed by LC-MS/MS were also analyzed by DART-MS. A successful identification was defined as the detection of cocaine and xylazine in all three replicates using the NIST/NIJ DART-MS Data Interpretation Tool.

To identify potential chemical background compounds from the test strip, an unused xylazine test strip was extracted using the optimized extraction method (Procedure 2). The extract was then analyzed by DART-MS. The resulting mass spectrum from the unused test strip was compared to the spectrum generated from the analysis of a used test strip extract from an authentic sample and to the spectrum from the analysis of the authentic sample using a traditional (non-test strip) substrate. Additional information about the collection and analysis of authentic samples is outlined next.

2.6 | Limit of Detection

The LOD study was completed using xylazine test strips. To establish an LOD, test strips were dipped into each concentration of the six-component solution set and the carfentanil solution set, in duplicate. The test strip was immersed in the solution until solution was visible in the test result area (shown in Figure 1). The test strip was then removed, allowed to dry, and extracted using the optimized approach (Procedure 2). Extracts were analyzed by DART-MS. The LOD was defined as the lowest concentration where the compound

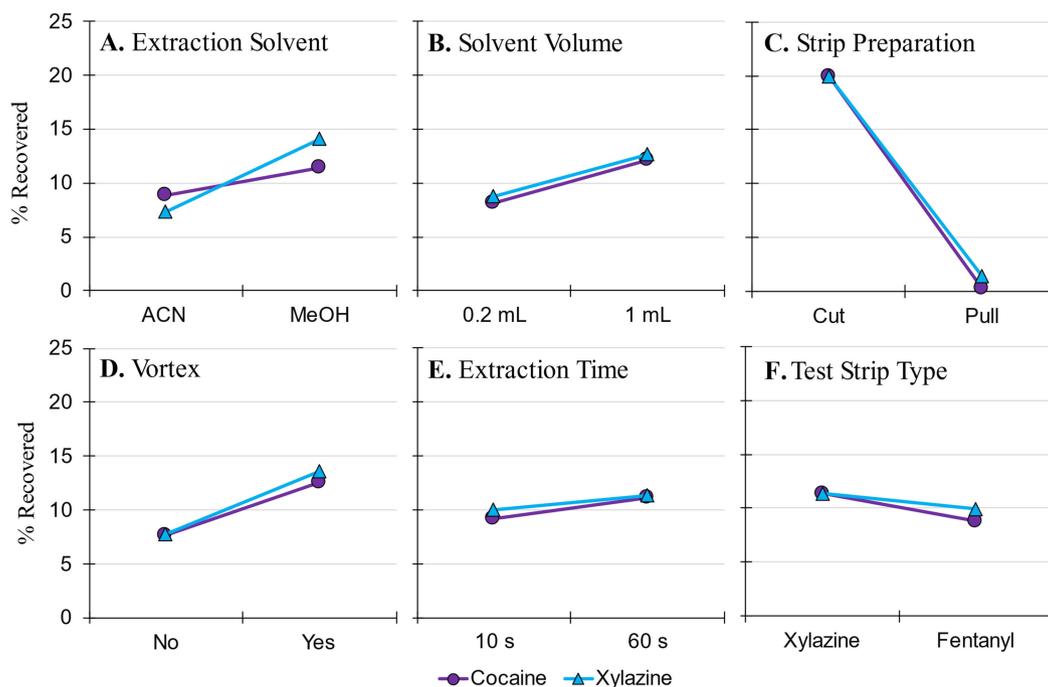


FIGURE 2 | Main effects plots for the extraction optimization study. The percent recovery for cocaine (dark purple circles) and xylazine (light blue triangles) are shown for each of the parameters studied.

of interest could be identified in both replicates using the NIST/NIJ DART-MS Data Interpretation Tool above a 3% relative intensity threshold and within a mass tolerance of $\pm 0.005 m/z$.

2.7 | Authentic Samples

To test the method using authentic samples, used test strips were submitted by three harm reduction sites (Sites 1–3), along with either drug product or trace residue collected from the same sample using the previously published methods [11]. Information was gathered from the sites on which test strips were used and how they were prepared (Table S2). After receiving the samples, test strips were extracted using the optimized method (Procedure 2) while the drug product or trace residue samples were prepared using a pre-existing method (See the Supporting Information) [11]. Both sets of samples were analyzed by DART-MS.

2.8 | LC-MS/MS Sample Prep and Analysis

For the extraction optimization study, LC-MS/MS was used to quantitate the amount of cocaine and xylazine present in samples. To do so, an eight-point calibration curve was created gravimetrically from the cocaine/xylazine stock solution to obtain concentrations spanning from 0.01 to 30 $\mu\text{g}/\text{mL}$ (exact values shown in Table S1 with the calibration curves shown in Figure S1). Calibration and sample solutions were prepared for LC-MS/MS analysis by combining 0.1 mL of the 1- $\mu\text{g}/\text{mL}$

internal standard solution with 0.1 mL of the respective curve solution in a 2-mL glass vial containing a glass insert.

A Thermo UltiMate 3000 (Waltham, Massachusetts, United States) liquid chromatography system coupled to a Sciex QTrap 4000 mass spectrometer (Framingham, Massachusetts, United States) was used for all analyses. Detailed method parameters are provided in the Supporting Information including the MRM transitions, and corresponding internal standards, used for quantitative analysis (Table S7).

2.9 | DART-MS Analysis

To be practical for drug-checking programs, it is important that the extraction method must be applicable to multiple analysis techniques. DART-MS was chosen as it is currently used in laboratory-based drug-checking programs [11] and provides a way to obtain a rapid, comprehensive chemical profile of a sample and thus may be well suited for this application. Test strip extracts were directly analyzed by DART-MS without any additional sample preparation using a Bruker DART-SVP ion source (Billerica, Massachusetts, United States). A JEOL AccuTOF LC-4G mass spectrometer using JEOL msAxel software (JEOL, Peabody, Massachusetts, United States) was used for all analyses to obtain high-resolution mass spectra. Complete method parameters are provided in the previously published work [11]. Analysis of the resulting mass spectral data was completed using the NIST/NIJ DART-MS Data Interpretation Tool (v3) [13] using the Inchworm library [14]. Search parameters included a peak intensity threshold of 3% and a mass tolerance of $\pm 0.005 \text{ Da}$.

3 | Results and Discussion

3.1 | Optimization of Test Strip Extraction

Optimization of the extraction method was completed using test strips doped with a known mass of cocaine and xylazine, to allow for direct comparison of results. Cocaine and xylazine were chosen as target analytes as they were available in bulk amounts (to create the necessary stock solution), represent common compounds encountered in drug-checking scenarios, and would be of interest to detect in used test strips. Fentanyl and xylazine test strips were chosen because they are commonly encountered test strip types in drug-checking/harm reduction scenarios. The inclusion of xylazine test strips and xylazine as a test compound also enabled the understanding of the effect of extracting a compound that the test strip is targeting. Pipetting a small volume of solution directly onto the test strip (instead of the traditional approach of dipping a test strip into an aqueous solution) minimized variability in the amount of material adsorbed into the test strip.

The optimization process began by first identifying potential extraction solvents. Four solvents commonly used in laboratories performing drug checking were studied—acetonitrile, methanol, water, and a 50:50 water:methanol mixture. Test strips, doped with known amounts of cocaine and xylazine, were prepared, allowed to dry overnight, and extracted (using Procedure 1). The resulting extract was then analyzed by LC-MS/MS to quantify the amount of cocaine and xylazine present. Five xylazine test strips were doped and extracted using each solvent.

The results of this study are shown in Table 1. Use of acetonitrile and methanol resulted in approximately three to four times higher percent recovery for both cocaine and xylazine when compared to water or the water:methanol mixture. The percent relative standard deviation (% RSD) across replicates was relatively consistent among solvent types (4.9%–7.3% for cocaine and 7.8%–13.7% for xylazine), indicating that no single solvent was better at obtaining a higher extraction reproducibility. Because acetonitrile and methanol were found to perform similarly, both solvents were considered in the next set of experiments.

Once the solvent choices were reduced to two, a design of experiments approach was conducted to identify the effect of solvent and other parameters on the extraction process. A 2^{6-1} partial factorial design was used, resulting in 32 sets of parameters, each analyzed in triplicate. Beyond solvent, other parameters studied

included solvent volume, test strip preparation, whether or not the sample was vortexed, extraction duration, and test strip type. For each parameter, two levels were chosen. Methanol and acetonitrile were the two solvents studied, based on the aforementioned results. For solvent volume, 0.2 and 1 mL were selected to mimic scenarios where an analyst may choose to use a limited volume to maximize concentration or use sufficient solvent to cover the test strip adsorption pad and provide enough sample for multiple analyses. For test strip preparation, options referred to as cut and pull were studied. In the cut step, the test strip was simply trimmed to fit in the vial—representing a simple and quick approach to preparing a sample. For pull, the sample pad was removed, using tweezers, from the rest of test strip to increase direct contact with the solvent. This process was significantly more time consuming and tedious. For vortexing, the options were “yes” (samples vortexed at 1047 rad/s (10,000 rpm)) or “no.” For extraction duration, 10 and 60s were selected to investigate the effect of increased extraction times on extraction efficiencies. Finally, for test strip type, fentanyl and xylazine test strips were used to identify if differences in antibody would affect results and if the extraction process was impacted if the compound of interest (e.g., xylazine) matched the test strip type (e.g., xylazine test strip). The exact design that was used for this study is provided in Table S3.

The average concentration and percent recovered of cocaine and xylazine for each sample extract are shown in Table S4. The average concentration for each variable is shown in Table 2 and is plotted as a main effects plot in Figure 2. To account for the impact of solvent volume on concentration, results are displayed as percent recovered. The parameter that most affected the recovery of analytes was test strip preparation. Simply cutting the test strip (average percent recovery: cocaine—20.0% and xylazine—19.9%) was found to perform significantly better than pulling out the sample pad (average percent recovery: cocaine—0.3% and xylazine—1.4%). This could indicate that the actual analyte being extracted is coming from other parts of the test strip, such as the conjugate pad or membrane, although this was not studied further. From a large-scale implementation perspective, this was a welcomed result as it greatly simplifies the sample preparation process.

For solvent, there was approximately a twofold gain in average percent recovery for xylazine when using methanol (14.0%) versus acetonitrile (7.3%) and a less significant gain for cocaine (11.4% for methanol and 8.9% for acetonitrile). As expected, vortexing the sample increased recovery of both cocaine and xylazine, both by

TABLE 1 | Average extract concentration and percent recovered for cocaine and xylazine extracted from doped xylazine test strips using four different solvents.

| Solvent | Average concentration ($\mu\text{g/mL}$) | | Average percent recovered (%) | |
|----------------------|--|------------------|-------------------------------|-------------------|
| | Cocaine | Xylazine | Cocaine | Xylazine |
| Acetonitrile | 10.6 (\pm 0.7) | 7.5 (\pm 0.6) | 36.5 (\pm 2.4) | 16.3 (\pm 1.3) |
| Methanol | 9.1 (\pm 0.7) | 9.1 (\pm 0.9) | 31.4 (\pm 2.3) | 20.5 (\pm 2.0) |
| Water | 1.8 (\pm 0.1) | 1.9 (\pm 0.3) | 6.1 (\pm 0.4) | 4.2 (\pm 0.6) |
| 50:50 Water:Methanol | 2.8 (\pm 0.1) | 3.0 (\pm 0.3) | 9.7 (\pm 0.5) | 6.7 (\pm 0.7) |

Note: Uncertainties represent the standard deviation of five measurements.

TABLE 2 | Average percent recoveries for each variable for cocaine and xylazine extracted from doped test strips.

| Variables | | Average percent recovered (%) | |
|--------------------|----------|-------------------------------|----------|
| | | Cocaine | Xylazine |
| Extraction solvent | ACN | 8.9 | 7.3 |
| | MeOH | 11.4 | 14.0 |
| Solvent volume | 0.2 mL | 8.2 | 8.7 |
| | 1.0 mL | 12.2 | 12.5 |
| Strip preparation | Cut | 20.0 | 19.9 |
| | Pull | 0.3 | 1.4 |
| Vortex | No | 7.8 | 7.7 |
| | Yes | 12.6 | 13.6 |
| Extraction time | 10 s | 9.2 | 10.0 |
| | 60 s | 11.2 | 11.3 |
| Test strip type | Xylazine | 11.5 | 11.3 |
| | Fentanyl | 8.9 | 10.0 |

Note: Results for each sample is shown in Table S3, and the results are plotted as a matrix plot in Figure 2.

roughly twofold. Other parameters showed more minor differences. The extraction efficiencies were nearly identical for 10 and 60 s, demonstrating that there is likely minimal to no gain in pursuing lengthy extractions. Test strip type also played a minimal role. Interestingly, the percent recovery for xylazine was higher using the xylazine test strip than the fentanyl test strip, indicating that there is more than enough unbound xylazine present on the xylazine test strip for extraction. The increase in xylazine percent recovery from the xylazine test strips could also be contributed to the binding of xylazine to the test strip that is then reversed during the extraction. Sample extracts with all the variables selected for the optimized method (ignoring solvent volume and test strip type) are bolded in Table S4.

For solvent volume, percent recovery was slightly higher for both analytes when using 1 mL. This is likely attributed to increased interaction between the solvent and the test strip, because dispensing 0.2 mL of solvent in a 2-mL vial meant that much of the test strip did not interact with solvent, especially when the sample was not vortexed. Therefore, a follow-on solvent volume study was completed to (1) determine if the lower recoveries for the 0.2-mL volume were caused by less interaction of the solvent with the surface of the test strip and (2) determine if an intermediate solvent volume, 0.5 mL, would produce similar recoveries to a 1-mL solvent volume. The driver for studying the 0.5-mL solvent volume was to determine if solvent consumption could be reduced while also maintaining a sufficient amount of solution for analytical measurements. To understand if the 0.2 mL solvent volume was driven by low interaction, 250- μ L glass inserts were added to the 2-mL vials to minimize the unoccupied space. However, when using an insert, the test strip could not be cut in the same location used for previous experiments; therefore, two sets of samples were created. Samples cut at Location 2

(Figure 1) were used to compensate for this and were studied for volumes of 0.2 (with and without an insert), 0.5, and 1 mL. Test strips cut at Location 1 (Figure 1) were studied for volumes of 0.2 (without an insert), 0.5, and 1 mL. The other parameters were held constant using the results from the DOE—methanol as the extraction solvent, cutting the test strip, vortexing the sample, and an extraction time of 10 s. The entire process was repeated in triplicate for both xylazine and fentanyl test strips.

Table 3 shows the average concentration and percent recovery for the follow-on solvent volume study for both cocaine and xylazine. The highest concentration values were recorded for the 0.2-mL solvent volume cut at Location 2, regardless of whether an insert was used. The highest percent recovery occurred when using 1 mL of solvent and cutting the xylazine and fentanyl test strips at Location 1 (46.5% and 38.6% for cocaine and 39.6% and 35.5% for xylazine). The 1-mL extraction volumes, however, lead to low-average extract concentrations, which was not unexpected. Ultimately, the 0.5-mL extraction volume was chosen as the slight reduction in percent recovery was balanced by an increased extract concentration, which was critical for ensuring low-level detection of compounds in a mixture.

The final, optimized extraction procedure, which was used for all the remaining parts of this study, was to cut the test strip (at Location 1, Figure 1) into a 2-mL vial, add 0.5 mL of methanol, cap the vial, vortex the sample for 10 s, and then remove the test strip to prevent readsorption.

3.2 | DART-MS Analysis

The goal of this project was to develop a method so that test strips could be submitted to drug-checking programs for additional analyses. Therefore, it is important that the optimized extraction method allows for the detection of compounds using various instrumentation. We studied the ability for test strip extracts to be analyzed by DART-MS as it represents a commonly employed tool that does not have a chromatographic separation component—making it more susceptible to influences from chemical background. To establish feasibility to analyze these extracts by DART-MS, the extracts created from the DOE study were also analyzed, and the resulting mass spectra were searched against the NIST DART-MS Forensics Database (version Inchworm) [14] within the NIST-NIJ DART-MS Data Interpretation Tool (v3) [13]. A successful identification was determined when the protonated molecule of both cocaine and xylazine could be identified above the 3% peak threshold and within a ± 0.005 Da mass tolerance. Xylazine was identified in all samples, and cocaine was identified in all but seven samples (one replicate of Run 31 and all three replicates of Runs 7 and 23, Table S3). For the samples that used the optimized extraction method (methanol as the extraction solvent, cut test strip preparation, vortexing, and an extraction length of 10 s), both cocaine and xylazine were always detected.

The second part to establish feasibility was to understand what chemical background is produced by extracting test strips and whether it would create false positive identifications. To do so, an unused test strip was extracted using Procedure 2 and then analyzed by DART-MS. The resulting mass spectrum (Figure 3A) highlights a number of ions that are attributed

TABLE 3 | Average extract concentration and percent recovered for the follow-on solvent volume studies.

| Compound | Volume (mL) | Insert in vial | Cut location | Average concentration ($\mu\text{g/mL}$) | | Average percent recovered (%) | |
|---------------------|-------------|----------------|--------------|--|-------------------|-------------------------------|-------------------|
| | | | | Cocaine | Xylazine | Cocaine | Xylazine |
| Xylazine test strip | 0.2 | Yes | 2 | 33.8 (\pm 0.6) | 46.1 (\pm 1.7) | 23.3 (\pm 0.4) | 20.7 (\pm 0.8) |
| | 0.2 | No | 2 | 35.4 (\pm 3.1) | 47.3 (\pm 4.5) | 24.4 (\pm 2.1) | 21.2 (\pm 2.0) |
| | 0.5 | No | 2 | 20.7 (\pm 1.2) | 22.3 (\pm 2.3) | 35.7 (\pm 2.0) | 25.1 (\pm 2.6) |
| | 1.0 | No | 2 | 11.2 (\pm 1.6) | 24.5 (\pm 4.3) | 38.5 (\pm 5.6) | 22.2 (\pm 9.6) |
| | 0.2 | No | 1 | 25.0 (\pm 8.0) | 29.8 (\pm 9.6) | 17.2 (\pm 5.5) | 13.4 (\pm 4.3) |
| | 0.5 | No | 1 | 21.3 (\pm 4.8) | 26.2 (\pm 6.5) | 36.7 (\pm 8.3) | 29.5 (\pm 7.3) |
| | 1.0 | No | 1 | 13.5 (\pm 0.3) | 17.6 (\pm 0.7) | 46.5 (\pm 1.0) | 39.6 (\pm 1.6) |
| Fentanyl test strip | 0.2 | Yes | 2 | 32.0 (\pm 0.3) | 51.1 (\pm 2.5) | 22.1 (\pm 0.2) | 23.0 (\pm 1.1) |
| | 0.2 | No | 2 | 30.4 (\pm 4.2) | 49.1 (\pm 6.7) | 21.0 (\pm 2.9) | 22.1 (\pm 3.0) |
| | 0.5 | No | 2 | 12.8 (\pm 0.4) | 21.0 (\pm 1.0) | 22.1 (\pm 0.6) | 23.6 (\pm 1.1) |
| | 1.0 | No | 2 | 8.2 (\pm 0.5) | 15.2 (\pm 3.7) | 28.2 (\pm 1.8) | 34.2 (\pm 8.3) |
| | 0.2 | No | 1 | 24.0 (\pm 3.4) | 32.7 (\pm 6.0) | 16.6 (\pm 2.4) | 14.7 (\pm 2.7) |
| | 0.5 | No | 1 | 19.3 (\pm 0.9) | 27.0 (\pm 2.7) | 33.4 (\pm 1.5) | 30.4 (\pm 3.1) |
| | 1.0 | No | 1 | 11.2 (\pm 0.4) | 15.8 (\pm 0.6) | 38.6 (\pm 1.5) | 35.5 (\pm 1.3) |

Note: Uncertainties represent the standard deviation of three measurements.

to background from the test strip, including peaks at nominal m/z 133, m/z 173, m/z 249, m/z 255, m/z 259, m/z 265, and m/z 355.

The mass spectrum of the unused test strip extract was searched using the aforementioned stated parameters to identify what, if any, false detections could be generated from this background. The peak at nominal m/z 265 produced a hit for the protonated molecule of tetracaine, a local anesthetic found in many fentanyl samples, although the resulting reverse match factor (RevMF) was quite low (0.546 a.u. on a scale of 0 a.u. (complete non-match) to 1 a.u. (complete match)). The peak at nominal m/z 249 produced a hit for the protonated molecule for pindolol, a blood pressure medication, with reasonable comparison scores (0.891 a.u. for FPIE and 0.990 a.u. for RevMF). Several other potential matches were identified, but both search score metrics were sufficiently low ($<$ 0.6 a.u.) to rule out potential false positive identification.

The mass spectrum of the unused test strip extract was also searched using an integer resolution m/z tolerance to mimic data that would be collected from a low-resolution mass spectrometer. This approach, as expected, increased the number of potential compounds identified, although the search scores for those identified remained quite low. Peaks that produced potential compound identifications with reasonably high ($>$ 0.7 a.u.) search scores included methyl-2-phenylacetate (m/z 133) and dimethylsulfone (m/z 95). The presence of these background peaks should not preclude the use of test strip extracts as a way to analyze samples, but it does highlight that caution needs to be taken to understand the chemical background of test strips prior to undertaking this approach.

3.3 | Limit of Detection

Once it was demonstrated that the detection of compounds extracted from used tests strips was feasible by DART-MS, the next component of this study sought to establish the approximate limits of detection for a panel of seven drugs—carfentanil, cocaine, fentanyl, heroin, isotonitazene, methamphetamine, and xylazine. The panel compounds were chosen to represent commonly detected drugs (cocaine, fentanyl, heroin, methamphetamine, and xylazine) as well as compounds assumed to be present at low mass fractions within samples (carfentanil and isotonitazene). Approximate LODs were obtained by dipping test strips into the solutions of decreasing known concentrations for the seven-drug panel (six-component solution and one-component solution) allowing the test strip to dry overnight, extracting using the optimized method, and analyzing the extract using DART-MS. Because both the fentanyl and xylazine test strips were found to have similar percent recoveries, only the xylazine test strips were used for this portion of the study. The approximate LOD was defined as the lowest concentration where the protonated molecule of the compound of interest could be identified using the 3% relative intensity threshold and \pm 0.005 m/z mass tolerance for both replicates. The reported concentrations correspond to the concentration of the aqueous solution that the test strip was dipped into.

The approximate LODs were either 5 or 50 $\mu\text{g/mL}$ (Table 4). To determine the operational relevance of these detection limits, corresponding minimal detectable mass fractions in a mixture were estimated. Estimates were made based on dissolving 5 mg of drug powder in 5 mL of water [15]. This approach results

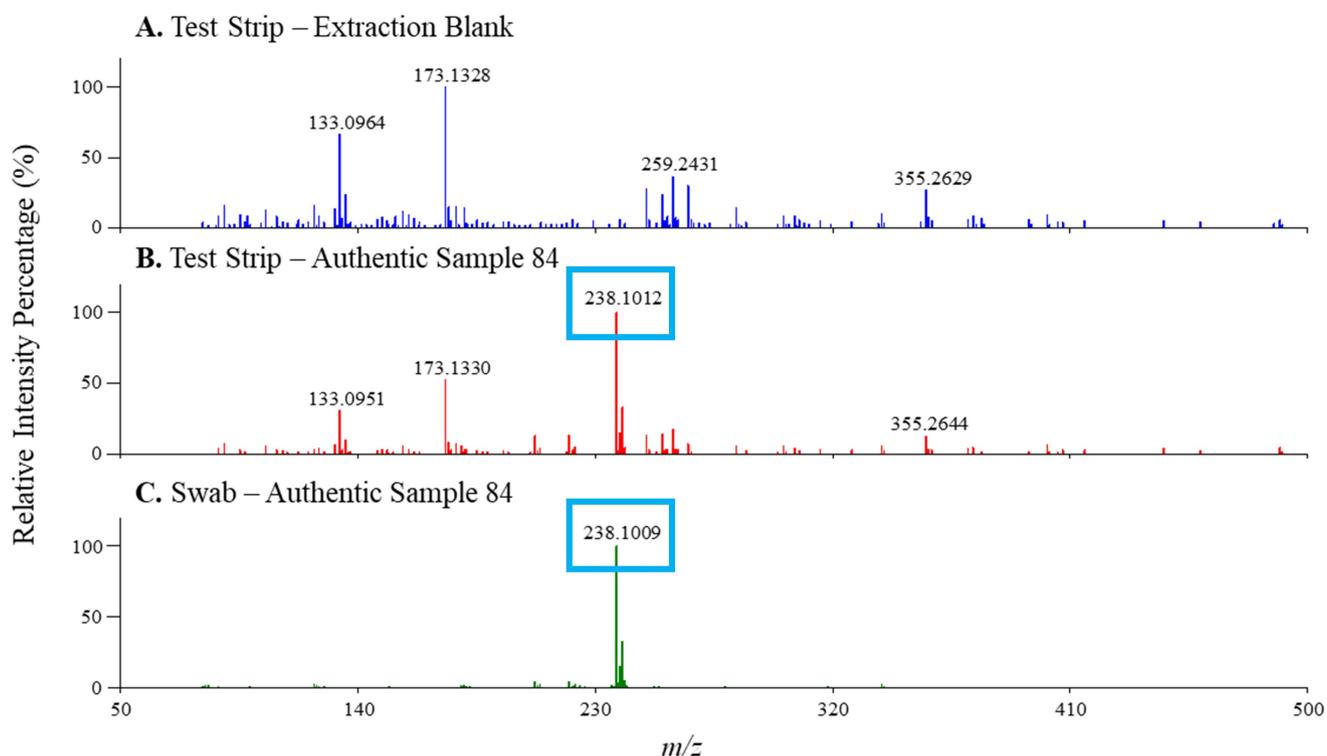


FIGURE 3 | Representative DART-MS spectra of an unused test strip extract: (A) the extract from a test strip associated with Authentic Sample 84 with ketamine detected at nominal m/z 238 highlighted by the blue box and (B) the extract from the corresponding trace residue collection of Authentic Sample 84 with ketamine detected at nominal m/z 238 highlighted by the blue box.

TABLE 4 | Approximate LODs for the seven drugs.

| Compound | Theoretical protonated molecule (m/z) | Approximate LOD ($\mu\text{g/mL}$) | Approximate lowest detectable mass fraction in a mixture ^a |
|-----------------|---|--------------------------------------|---|
| Carfentanil | 395.233 | 50 | 0.05 (5%) |
| Cocaine | 304.155 | 5 | 0.005 (0.5%) |
| Fentanyl | 337.228 | 5 | 0.005 (0.5%) |
| Heroin | 370.165 | 50 | 0.05 (5%) |
| Isotonitazene | 411.240 | 50 | 0.05 (5%) |
| Methamphetamine | 150.128 | 5 | 0.005 (0.5%) |
| Xylazine | 221.111 | 5 | 0.005 (0.5%) |

Note: Also listed in the table are the approximate mass fractions within a mixture corresponding to the LODs, assuming a standardized sample preparation of adding approximately 5 mg of powder to 5 mL of water (1 mg/mL).

^aApproximate detectable mass fraction assumes that 5 mg of drug powder is mixed in 5 mL of water, producing a solution with a total concentration of 1000 $\mu\text{g/mL}$. The mass percent of total weight is shown in parentheses.

in a total solution concentration of approximately 1000 $\mu\text{g/mL}$, translating to 0.005 mass fraction (0.5% of total weight) detection capability for an LOD of 5 $\mu\text{g/mL}$ and a 0.05 mass fraction (5% of total weight) detection capability for an LOD of 50 $\mu\text{g/mL}$. It is presumed that carfentanil and isotonitazene are found at low mass fraction in a mixture given their increased potency relative to fentanyl [16, 17]. Therefore, it is possible that this approach would miss the detection of these compounds. Solution concentrations could be increased by dissolving more drug powder or using a lower volume of water to increase the detection probability of these important compounds.

3.4 | Evaluation of Extraction Approach Using Authentic Samples

The final component of this study was focused on evaluating the applicability of the optimized extraction procedure using authentic samples. To do so, three drug-checking sites (two from the West Coast and one from the East Coast of the United States) submitted used test strips to the laboratory along with either drug product (submitted as several milligrams of powder in a vial containing acetonitrile) or as trace residue (collected using either a swab or a wipe) [11]. The sites were asked to prepare solutions for test strip

analysis as they normally would (see Table S2 for detailed procedures), use any combination of test strips they felt necessary, and after using the test strips, mail them, along with the wipe, swab, or vial, to the laboratory for testing. In total, 105 unique samples were submitted, each containing at least one test strip. Of the 105 samples, 14 had three used test strips per sample, and five had four used test strips per sample, which also enabled us to investigate whether consistent results could be obtained from different test strip types. In total, 148 used test strips were received.

To determine how well results from drug product or trace residue collection matched those from a used test strip, the test strips were extracted using the optimized procedure and analyzed qualitatively using DART-MS. Wipes, swabs, and vials were prepared using the previously established methods (See the [Supporting Information](#)) [11] and analyzed using DART-MS. The resulting mass spectra from both the test strip(s) and the corresponding sample were then searched using the previously discussed parameters. Identifications for all spectra were made and assigned to one of the three categories—(1) compound detected in both test strip extract and corresponding sample, (2) compound detected in only the corresponding sample, or (3) compound detected in only the test strip extract. An example test strip extract and corresponding sample mass spectrum are provided in Figure 3 for Sample 84, where ketamine (protonated molecule at nominal m/z 238) was identified in both spectra. Complete results for this component of the study are provided in Table S5.

First, the ability to obtain results that were in concordance with the test strip result was evaluated. Concordance was obtained when either (1) the extract from a test strip with a positive result contained a test strip target compound (e.g., heroin was detected in an extract from an opiate test strip with a positive result) or (2) the extract from a test strip with a negative result did not contain test strip target compounds (e.g., heroin or another opiate was not detected in an extract from an opiate test strip with a negative result). Non-concordant results were obtained when either (1) the extract from a test strip with a positive result did not contain a target test strip compound (e.g., heroin or another opiate was not detected in an extract from an opiate test strip with a positive result) or (2) the extract from a test strip with a negative result did contain a target test strip compound (e.g., heroin was detected in an extract from an opiate test strip with a negative result).

As outlined in Table 5, concordant results were obtained 95.9% of the time, with the vast majority (80.4%) occurring because of concordant negative results. Of the six instances where non-concordant results were obtained, only one was due to the detection of a compound in the test strip extract that should have resulted in a positive test strip result. This sample (Sample 26, Table S6) was found to contain xylazine, even though the xylazine test strip response was negative. The xylazine peak in the

DART-MS mass spectrum was quite low (approximately 3% relative intensity), indicating that it may have been below the detection limit of the test strip. The remaining five non-concordant results resulted from the lack of the test strip target analyte being detected in the extract of a positive test strip. In four instances, (Samples 91, 99, and 102—two test strips, Table S6) fentanyl was not detected in the extract from a positive fentanyl test strip, and in one instance (Sample 96, Table S6), a benzodiazepine was not detected in the extract from a positive benzodiazepine test strip. This is likely attributed to a very low concentration of fentanyl or a benzodiazepine in the drug mixture. In Sample 91, fentanyl was not detected in the extract from the trace residue (wipe collection) either, likely indicating that a trace amount fentanyl was present in the original sample. For Samples 99 and 102, fluoro-fentanyl (Sample 99) and fentanyl (Sample 102) were detected in the drug product (Sample 99) and trace residue (swab collection, Sample 102) that were provided, indicating that a fentanyl was present at a level likely just below the LOD of the test strip extraction method. Trace levels of a benzodiazepine in Sample 96 are likely responsible for the missed detection, because the provided drug product sample was also found to not contain a detectable benzodiazepine. While a high degree of concordance was obtained between the test strip and test strip extract, it is important to note that there is the potential for low-level compounds to be undetectable in the extract because they are present at a concentration below the instrument LOD.

While concordance is an important metric to understand, the key purpose of this approach is to detect other compounds in the drug mixture that would not be represented by the test strip result. This was completed by evaluating the results obtained from the test strip extracts and comparing them to the results of the wipe, vial, or swab collected from the same sample. In the 148 test strips and corresponding samples, there were a total of 342 unique compound identifications (a compound detected in either the test strip extract, corresponding sample, or both). Two of the test strips—corresponding sample pairs—exhibited no compounds detected in either extract.

The results of this portion of the study were used to answer five questions:

1. How well are compounds detected in test strip extracts that are present in the wipe, vial, or swab extract?
2. Are compound matches better when comparing test strip extracts to results obtained from a drug product or a trace residue collection (either a wipe or a swab)?
3. Does the type of test strip used affect the ability to identify compounds?
4. Does the method of test strip preparation affect the ability to identify compounds?

TABLE 5 | Concordance results demonstrating an agreement between the visual test strip result and the test strip extract result.

| | Positive test strip | Negative test strip |
|---|---------------------|---------------------|
| Test strip target compound(s) identified in extract | 23 (15.5%) | 1 (0.7%) |
| Test strip target compound(s) not identified in extract | 5 (3.4%) | 119 (80.4%) |

Note: A total of 148 test strip results were obtained from 105 corresponding samples.

5. Are there compounds that are more difficult to detect in test strip extracts, and if so, why?

Globally, approximately 79% of the 342 unique compound identifications were the result of the compound being detected in both the test strip extract and the corresponding sample (Table 6). There were 49 instances (14%) where a compound was detected in the corresponding sample but not in the test strip extract, which was expected given the previously described limitations of compound detection near the LOD of the approach. It is also possible that sample heterogeneity could affect these results. Interestingly, there were also 23 instances (7%) where the test strip extract contained a compound that was not detected in the corresponding sample. This was attributed to the method by which the corresponding sample was received, that is, as a drug product or as a trace residue. All 23 instances were from trace residue samples (18 swabs and 5 wipes). Given that many of the trace residue samples come from used paraphernalia (e.g., syringes, baggies, and cookers), it is expected that an incomplete chemical profile could be obtained because of low collection efficiencies of minor components. For the drug product samples, there were no instances where the test strip extract led to a compound detection not found in the sample, which was also expected given the higher concentrations present in these samples. There were four instances (29% of drug product samples) where a compound was identified in the sample and not the test strip extract, which is also likely due to the ability to detect lower concentration

compounds in the drug product because of higher starting masses of materials.

Interestingly, neither the type of test strip used nor how the test strip solution was prepared played a major role in how well compound identifications matched (Table 6). Excluding amphetamine test strips, because of the low number of strips provided, compound matches ranged from 70% to 90% across test strip types. The opiate test strips were found to perform slightly worse (70% compound matches) than other test strip types. Fentanyl and xylazine test strips, which are most commonly used in drug-checking scenarios, performed almost identically and were in line with the global average of approximately 80% compound matches. The test strip solution preparation varied from site to site ranging from 0.5 mg/mL (Site 3) to 4 mg/mL (Site 1) as well as the use of different test strip manufacturers. Even considering the difference in test strip solution preparation, the compound matches were similar, ranging from 74% (Site 2) to 81% (Sites 1 and 3) and agreed with the global average of approximately 80% compound matches.

Finally, there does not appear to be any compound-specific trends related to the ability to be detected in the test strip extract (Table S5). All compounds, except fluorofentanyl, were detected in both the test strip extract and the corresponding sample greater than 50% of the time, with the majority being detected in both more than 80% of the time. This is promising with regard to the ability to confidently extract a wide range of

TABLE 6 | Summary results for the comparison between the test strip extract results and the corresponding sample results organized by test strip type, test strip preparation approach, and the type of corresponding sample.

| | Number of unique compound IDs | Compound detected in both test strip extract and corresponding sample | Compound detected in test strip extract only | Compound detected in corresponding sample only |
|---|-------------------------------|---|--|--|
| Overall | 342 | 270 (79%) | 23 (7%) | 49 (14%) |
| Test strip type | | | | |
| Amphetamine | 4 | 4 (100%) | 0 | 0 |
| Benzodiazepine | 150 | 116 (77%) | 15 (10%) | 19 (13%) |
| Fentanyl | 79 | 61 (77%) | 2 (3%) | 16 (20%) |
| Methamphetamine | 10 | 9 (90%) | 0 | 1 (10%) |
| Opiate | 20 | 14 (70%) | 2 (10%) | 4 (20%) |
| Xylazine | 79 | 66 (84%) | 4 (5%) | 9 (11%) |
| Test strip preparation/provider | | | | |
| Site 1 | 130 | 105 (81%) | 14 (11%) | 11 (9%) |
| Site 2 | 89 | 66 (74%) | 5 (6%) | 18 (20%) |
| Site 3 | 123 | 99 (81%) | 4 (3%) | 20 (16%) |
| Type of corresponding sample received with test strip | | | | |
| Drug product (vial) | 14 | 10 (71%) | 0 | 4 (29%) |
| Trace residue (swab) | 254 | 205 (81%) | 18 (7%) | 31 (12%) |
| Trace residue (wipe) | 74 | 55 (74%) | 5 (7%) | 14 (19%) |

compound types from different drug classes. Some compounds, like heroin and mannitol, which have poorer detection limits on DART-MS than other compounds, did exhibit some of the lower percentages in terms of detection in both the test strip and the corresponding extract.

Perhaps to be expected, it appeared that the concentration of the compound in the sample was more important than the compound identity. While the way DART-MS analyses were conducted is not quantitative in nature, the relative abundance of the peaks can loosely translate to relative concentration of the compounds in the samples. Looking at the relative intensity of compounds that were detected in the corresponding sample but not the test strip extract (Table S5), intensities of the missed compounds were typically less than 15%. This is corroborated by the LOD component of this study, which highlighted that the detection of compounds at lower concentrations, when using test strip extracts, is likely not possible.

4 | Conclusions

As fatal and nonfatal drug overdoses continue to remain near all-time highs and the drug landscape continues to rapidly change, tools and approaches to readily analyze drug contents remain a major need. While on-site, comprehensive testing remains the ideal, there are often financial, logistical, and legal barriers preventing this approach. Low-barrier testing using fentanyl and xylazine test strips remains a dominant way for people who use drugs to better understand the composition of their samples. Results obtained from these tests are limited (e.g., xylazine is or is not present in a sample), but the method of sample collection enables post-collection analysis that could be used to obtain a more comprehensive picture.

In this study, we demonstrated the viability of extracting drug residues from used test strips as a potential mechanism to obtain low-barrier samples to better characterize the drug landscape. To do so, we first established an optimized protocol for the extraction of drug residues from used drug-checking test strips. The optimized method consisted of extracting analytes of interest from a cut test strip using 0.5-mL methanol while vortexing for 10s. The protocol was optimized using cocaine and xylazine as target analytes. Subsequent studies investigating the approximate LOD revealed that extraction from test strips followed by DART-MS analysis resulted in the ability to detect components down to a mass fraction of 0.005 (0.5% of total weight) in a mixture. Extraction of used test strips from authentic samples demonstrated greater than 96% concordance with the test strip result. Comparing test strip extract results to extracts of wipes or cotton swabs from the same sample resulted in approximately 80% agreement in compound identification. This was promising given that the preparation of the test strip solution, type of test strip used, and method of test strip analysis were uncontrolled. Test strips were found to contain a high chemical background, which led to an overall reduction in peak intensity for peaks of interest in the DART-MS mass spectra and likely hindered some of the low-level detection of compounds.

Although this study shows promise for the use of test strips to obtain comprehensive chemical information about a drug sample, there are several limitations that warrant future study. First, optimization of the test strip extraction process was only completed on one brand of test strip and only two types of test strips. While the authentic sample analysis component of this study showed that this approach worked on other types of test strips, thoroughly investigating the extraction approach on other test strips could be a fruitful venture for future work. Second, this work focused solely on the extraction component and did not evaluate the best method of sample preparation (e.g., how much powder and water, or other solvent, should be used to prepare the initial mixture for test strip analysis) to maximize the ability to extract and detect low-level compounds in drug mixtures. Third, while we were able to demonstrate the ability to qualitatively detect the same compounds in test strip extracts as would be detected in actual drug samples, a large number of these comparisons used trace residue collections and not actual bulk drug product. The use of trace residue does result in sample heterogeneity and cross-contamination possibilities that would be eliminated by further studies using solely bulk drug product. Fourth, the comparison with authentic samples completed in this study used DART-MS as the analytical platform. While DART-MS is appealing for its ability to generate high-quality data rapidly, the lack of chromatographic separation presents some unique challenges. Given the high chemical background from the test strips, it is possible that this type of analysis is better suited for a non-targeted liquid chromatography mass spectrometry approach. A comparison between analytical platforms to investigate this would help inform the best approach for analysis. Finally, a comprehensive study on used test strip storage and shipment should be completed to understand what effect these factors have on the ability to extract from test strips. Collection dates were provided by the submitting agencies for each sample, and the time from collection to analysis spanned from a couple of days to several weeks. There were no major deleterious effects due to storage and shipment, but this was not studied in a rigorous fashion.

As drug-checking efforts continue to increase across the United States, the ability to provide rapid, comprehensive information about the drug landscape will continue to remain a need. While completing comprehensive analytical testing for the drug product will be the ideal, this work demonstrates that there is an opportunity to gain more comprehensive chemical information from low-barrier test strips. This approach could allow people who used drugs, public health and law enforcement officials, policymakers, and the public a way to gain increased insights into the drug landscape without requiring significant capital investment.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

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

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.