The Final Step Towards Automated LC-MS Screening in Forensic Toxicology

Implementation of Online Sample Preparation by microSPE for Urine Drug Screening

Abstract

The throughput and reliability required for systematic toxicological analysis (STA) in modern toxicology laboratories today are more and more reliant on automation for sample handling, processing, and generation of results in timely, cost-effective workflows. Although the analytical benefits of LC-MS screening of biological samples are well established, online sample preparation for fully automated screening has not yet been demonstrated. An online application of micro solid phase extraction (µSPE) has been developed and evaluated for automated preparation of urine samples prior to LC separation and MS analysis. The procedure was evaluated against traditional protein precipitation (PP) in the determination of target limits of detection (LODs) in spiked urine pools and via the (re)screening of archived subject samples using the Toxtyper® LC-MS^n workflow. Results indicated that the combination of online µSPE with the Toxtyper® screening workflow is suitable for reproducible and efficient automated STA.

Keywords: forensics, automated sample preparation, Toxtyper®, TargetScreener, Elute HT

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Introduction

The detection and identification of all substances of toxicological relevance in biological samples is a central task in many of today's forensic toxicology laboratories, and such systematic toxicological analysis (STA) can employ a variety of analytical techniques. Many target compounds (e.g., alcohol, poisons, therapeutic drugs, and drugs of abuse, as well as many of their metabolites) have a great diversity of chemical characteristics, and the structural similarities among others (e.g., designer opioids) can challenge the discriminatory power of common screening approaches. The nature of the bodily fluids commonly tested can also present difficulties, whether by their complexity, as in blood-based samples, by broad concentration ranges, as in urine, or due to the requirements for sampling, as for oral fluid. Sample preparation prior to analysis is often mandatory to achieve the necessary sensitivity and quantitative accuracy in comprehensive sample screens.

Toxicology laboratories have often used high throughput immunoassays (IA) for routine screening of bodily fluids, benefiting from the advantages of speed and simplicity. Modern immunoassays are facilitated by a high degree of automation regarding sample preparation and reporting of results, supporting overnight services as well as 24/7 emergency toxicology analyses. The utility of immunoassays, however, can be limited by a number of factors. Many immunoassays target complete drug classes, e.g., amphetamines, benzodiazepines or opiates, and might require a second analytical method for compound identification. For a complete "panel screening," multiple tests are needed, each increasing analysis costs. The rapid appearance of new recreational drugs onto the market, particularly new psychoactive substances (NPS), requires the development of new antibodies, and the delay in their commercial availability may be significant, often leading to false negative reporting. False positive results are not uncommon due to cross-reactivity with other drugs or natural biogenous substances. Positive immunoassay results obtained in forensic-toxicological analyses usually require confirmatory analysis using an independent analytical method, often LC-MS/MS [1].

In recent years, LC-MS has become a key technique in STA. Both ion trap and QTOF based LC-MS screening offer wide-ranging compound detection and confident identification in a single analysis, with capabilities for fully automated data evaluation and reporting of screening results. In addition, such MS-based techniques offer unique analytical advantages, including the ability to rapidly expand databases and spectral libraries as new designer drugs appear on the market. This flexibility is valuable for both active (on-going) and retrospective analyses.

Although LC-MS offers considerable analytical power for comprehensive toxicological screens, appropriate sample preparation has been the missing piece towards fully automated

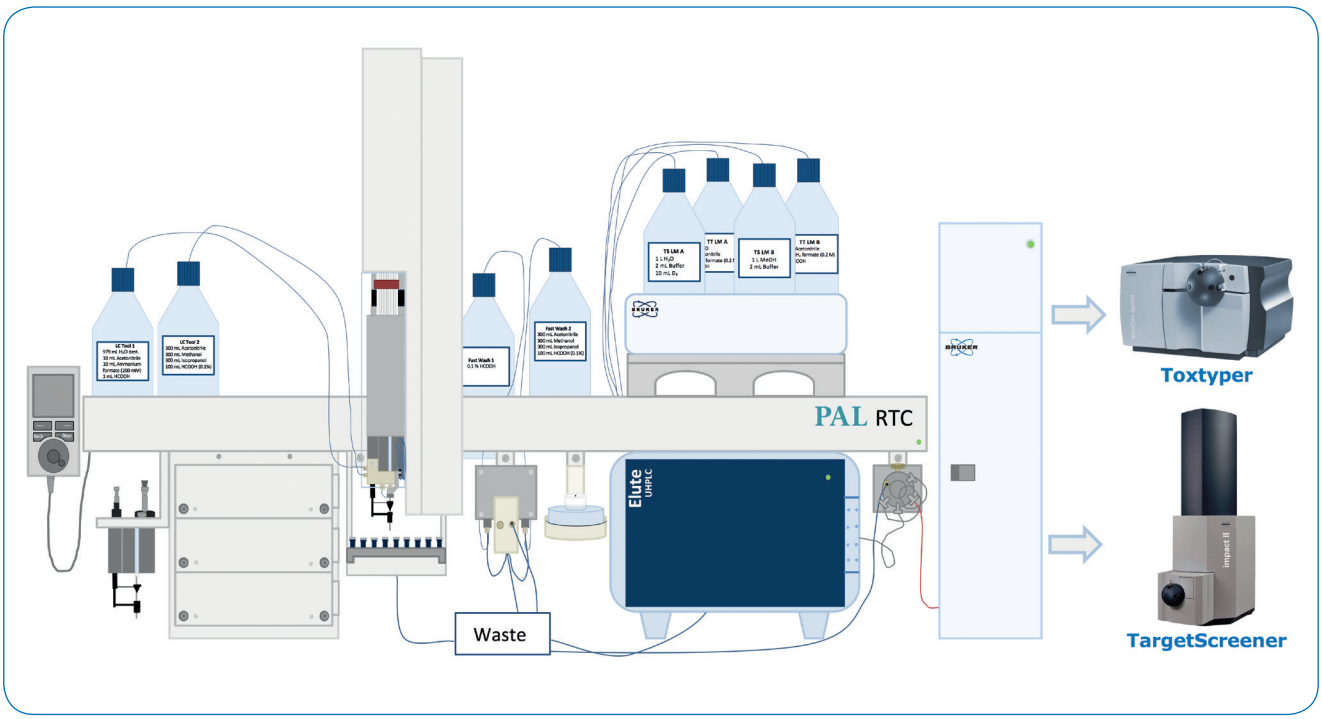


Figure 1: Experimental set up for automated workflow, including the sample handling system (CTC Analytics AG) and Toxtyper® or TargetScreener systems.

drug analysis. In this study, an online μSPE sample preparation method was developed and evaluated in combination with established LC-MS urine screening systems for routine forensic toxicology analysis. The Limits of Detection (LOD) were determined via online μSPE-LC-MS^n using the Toxtyper® and compared to standard manual sample preparation by protein precipitation (PP) with cold acetonitrile. Likewise, archived urine specimens from routine and post-mortem screenings were reanalyzed in parallel using μSPE-LC-MS^n and LC-MS^n following manual PP.

Experimental

Samples

Due to the diversity of urine samples used for this multi-faceted evaluation of online μSPE, the respective sample characteristics are described within each corresponding Results and Discussion subsection.

Protein Precipitation (PP)

100 μL of urine and 5 μL of a deuterated internal standard (ISTD) mix were pipetted into an Eppendorf tube, followed by 500 μL of cold (-20°C) acetonitrile (ACN, ≥99.9% purity, Honeywell, Seelze, Germany). An appropriate ISTD mix (D3-Morphine, D4-Risperidone, D4-Haloperidol, D5-Diazepam, and/or D5-MDMA) was used for all analyses. The sample was then mixed on a vortex mixer for three minutes, followed by centrifugation for five minutes at 13,200 RPM. The supernatant was transferred into an empty HPLC vial and evaporated to dryness under a gentle stream of N_2 at 40°C. The resulting residue was redissolved in 25 μL Toxtyper® mobile phase A:B, 50:50 (v/v) for Toxtyper® analysis.

Online μSPE HPLC-MS

A PAL RTC sample handling system (CTC Analytics AG, Zwingen, Switzerland) was used for sample preparation using C18-10 smartSPE™ μSPE cartridges (ITSP Solutions, Inc., Hartwell, GA, USA) [2], followed by direct injection into an Elute UHPLC (Bruker Daltonics) to continue through the standard automated Toxtyper® (amaZon speed ion trap MS system, Bruker Daltonics) workflow (Figure 1). Following initial testing of tools within the robotic sample handling system [3], a 250 μl LC-MS Tool was selected for all liquid handling, including the injection step.

The μSPE cartridges were washed with 0.2% ammonium acetate in MeOH and conditioned with 20% aqueous ammonium acetate. Afterward, 200 μL of urine was loaded onto the prepared cartridge and desalted with 20% aqueous ammonium acetate. Compounds were eluted with 75 μL 0.2% ammonium acetate in MeOH. The complete μSPE sample preparation time was 14.4 minutes. Washing procedures of the sample handling tool had been previously optimized to avoid any carry-over of sample or solvents during the entire extraction process. Further technical details regarding the online μSPE sample preparation have been outlined elsewhere [3]. LC and MS conditions were according to standard Bruker Toxtyper® protocols [4, 5], with a total run time of 11 minutes.

Results and Discussion

Limits of Detection (LOD)

A pooled blank urine sample (n=10) was spiked with 71 compounds found most in routine toxicology screens in the previous year. Four different concentrations were prepared at 100, 75, 50, and 25 ng/mL. All samples were analyzed in parallel using standard PP or C18-10 online μSPE, followed by LC-MS^n using the Toxtyper®. Analyses were made in triplicate, and the lowest concentration with automatic identification of each target in all three triplicates was set as the Limit of Detection (LOD) (Table 1).

Both sample preparation methods gave identical LODs for 42 of the tested substances. For 19 compounds, sample preparation carried out by online μSPE led to lower LOD values, while 10 compounds had lower LODs using protein precipitation. The LODs are suitable for STA in emergency and post-mortem toxicology, but insufficient for trace analysis as required for sobriety testing or analysis of drug-facilitated crime (DFC) cases.

The cocaine metabolite ecgonine methyl ester (EME) could not be detected using the μSPE workflow, whereas the LOD of the routine sample preparation via protein precipitation was found to be 50 ng/mL. In true forensic cases, this would not be a limitation, however, as benzoylecgonine, the major metabolite of cocaine, could be detected at concentration levels even sufficient for sobriety testing according to the CTU 3 criteria [6].

Enzymatic or chemical hydrolysis is often used before sample extraction in effort to improve the detection of certain metabolites of opiates/opioids and benzodiazepine which are prone to conjugation [7]. A cleavage step could be included within this robotic setup, however, the overall extraction time would be increased. Similarly, an increased volume of elution solvent would enhance the elution of compounds of interest but

Table 1: Limits of detection (LOD) for the Toxtyper® LC-MS^n screening workflow following sample preparation via standard PP or online µSPE

Equivalent LODs via µSPE and PP	
3-OH-Bromazepam	
Amlodipine	
7-Aminoclonazepam	
9-Hydroxyrisperidone	
Amisulpride	
Amitriptylinoxide	
α-OH-Midazolam	
Benzoylecgonine	
Bisoprolol	
Bromazepam	
Carbamazepine	
Clomipramine	
Clonazepam	
Cocaine	
Dextromethorphan	
Dihydrocodeine	
Doxepin	
Flunitrazepam	
Gabapentin	
Hydrocodone	
Hydroxyzine	
Ibuprofen	
Ketamine	
Lamotrigine	
Lorazepam	
LSD	
MDA	
Melperone	
Methadone	
Metoprolol	
Midazolam	
Morphine	
Norbuprenorphine	
Nordazepam	
Normorphine	
Nortilidine	
Nortriptyline	
Opipramol	
Pregabalin	
Ritalinic acid	
Sertraline	
THC-COOH	
U-47700	

Lower LODs via µSPE		
	µSPE	PP
4-Formylaminoantipyrine		
4-Methylaminoantipyrine		
7-Aminoflunitrazepam		
Ambroxol		
Amitriptyline		
Bupropion		
Codeine		
Codeine-6-glucuronide		
Desmethylmirtazapine		
Diphenhydramine		
Flupirtine		
MDEA		
MDMA		
Naloxone		
Nicotine		
Norfentanyl		
Oxycodone		
Pipamperone		
Trazodone		

Lower LODs via standard PP		
	µSPE	PP
Aripiprazol		
Buprenorphine		
Ecgonine methyl ester		
Fluoxetine		
Nordoxepine		
Paracetamol		
Paroxetine		
Promazine		
Temazepam		
THC-COOH-glucuronide		

>100 ng/mL	
100 ng/mL	
75 ng/mL	
50 ng/mL	
25 ng/mL	

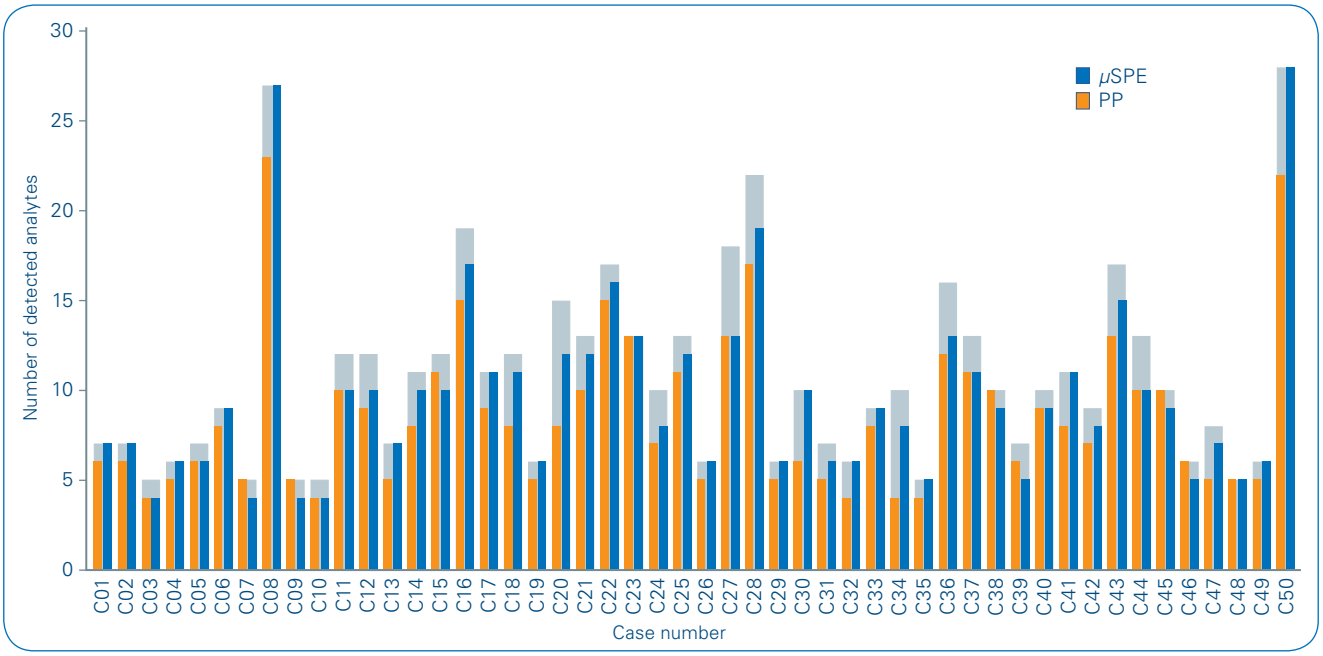


Figure 2: Number of detected analytes in true archived urine samples (n=50) using PP (orange) or online µSPE (blue). The sum of the compounds identified using PP and µSPE is displayed in grey.

would require the incorporation of an evaporation unit and subsequent reconstitution in a smaller volume of solvent. An evaporation unit would also provide more flexibility in the choice of elution solvents and µSPE chemistries, as the eluate need not be (directly) LC-MS compatible.

Analysis of True Forensic Samples

A set of 28 urine and 22 post-mortem urine samples obtained from real case work of the Institute of Forensic Medicine, Freiburg, Germany were (re)examined using the Toxtyper® after sample preparation via online µSPE-LC and standard PP (Figure 2). To assure the plausibility of the identified compounds, these results were compared to the original screens, which were previously carried out using routine analytical methods and documented in the toxicological and medico-legal reports. Unlike the fortified blank urine samples used for the initial method testing, the urine samples in true forensic toxicology show higher diversity in the number of analytes, metabolites, and matrix components. In particular, post-mortem samples may be severely altered, leading to challenges in sample preparation and analysis.

For the comparison, the sum of all different analytes identified by µSPE and PP and confirmed by routine analytical methods was set as 100%. Using C18-10 µSPE, 90% of the substances could be identified, whereas LC-MS^n screening after protein precipitation led to an identification of only 80% of the compounds.

Two common markers of alcohol consumption, ethyl glucuronide (EtG) and ethyl sulfate (EtS), were excluded from this evaluation. The detection and quantitation of these markers in true biological samples often require specific enrichment techniques, as their high polarity precludes the use of traditional reverse phase chemistries.

Notable differences among the screening methods were observed in some cases. In two cases, µSPE enables drug detection not possible by standard screening using PP. The antiparkinson drug pramipexole could be detected in one post-mortem case in both µSPE runs but not in the initial routine PP Toxtyper® screening, although it had been detected in the corresponding cardiac blood and vitreous humor. In a second case, amphetamine was found after µSPE but not in the routine PP Toxtyper® workflow. The amphetamine finding was confirmed by LC-QTOF-MS using TargetScreener.

In several cases, parent compounds and metabolites were simultaneously detected only using the online µSPE approach. The antidepressant citalopram, for example, could be identified in one case using both sample preparation methods, however, only µSPE led to additional identification of the metabolite esmethylcitalopram (Figure 4), confirming the uptake of citaloporam as well as prolonging the window of detection. The same was observed in the case of olanzapine and its metabolite n-desmethyl olanzapine.

Evaluation of reproducibility, matrix effects, and recovery via TargetScreener

Reproducibility

The consistency of experimental data obtained from an analytical method is an important parameter in forensic toxicology. The reproducibility of the complete online μSPE screening workflow was tested with a pooled urine sample (n=6) spiked with a set of 12 compounds of clinical relevance (3-hydroxybromazepam, α-hydroxy-alprazolam, bromazepam, fentanyl, MDMA, methadone, methamphetamine, morphine, morphine-3-β-D-glucuronide, nordiazepam, oxazepam, and zolpidem). Low (25 ng/mL) and high (100 ng/mL) concentrations were prepared and each of the samples was analyzed 10 times.

MS analysis was performed using TargetScreener. For data evaluation, the ratio of the absolute peak areas of analyte and ISTD (D5-MDMA) and the respective RSD_r [%] values were calculated. The RSD_{Low} for 11 of the 12 target compounds ranged from 5.6-10.9% and RSD_{High} (from 4.3-11.2% [3]). These relative standard deviations are lower than 15% and thus the approach is considered to be reproducible according to GTFCh guidelines for quantitative analysis [7]. However, morphine-3-β-D-glucuronide failed to meet this criterium (data not shown). As no glucuronide cleavage was performed prior to μSPE, its polarity and hydrophilicity resulted in poor retention on the C18-10 cartridge and consequently much higher RSDs.

Matrix Effects (ME) and Recovery (RE)

To evaluate matrix effects (ME) and recovery (RE), a protocol from Matuszewski et al. [8] was used with slight modifications. Twenty-two selected compounds (Figure 3) were spiked into six different urine samples at 27.5, 275, and 500 ng/mL. Two replicates of each sample were subjected to automated sample preparation using C18-10 μSPE cartridges, followed by TargetScreener LC-MS analysis. ME and RE were calculated by comparison with the analytical data obtained for the same compounds in neat mobile phase and blank urine samples that were spiked after extraction.

The average ME and RE of the 22 tested analytes are shown in Figure 3. The maximum ion suppression was approximately 50%, which is considered adequate for a screening approach. While ion suppression will have adverse effects on the LOD, ion enhancement is not an issue in screening approaches. Satisfactory recovery rates were achieved for the majority of the compounds. For morphine-glucuronide and the cocaine metabolite ecgonine methyl ester (EME), however, the overall yield was very low due to the low retention of these polar compounds on the C18 material.

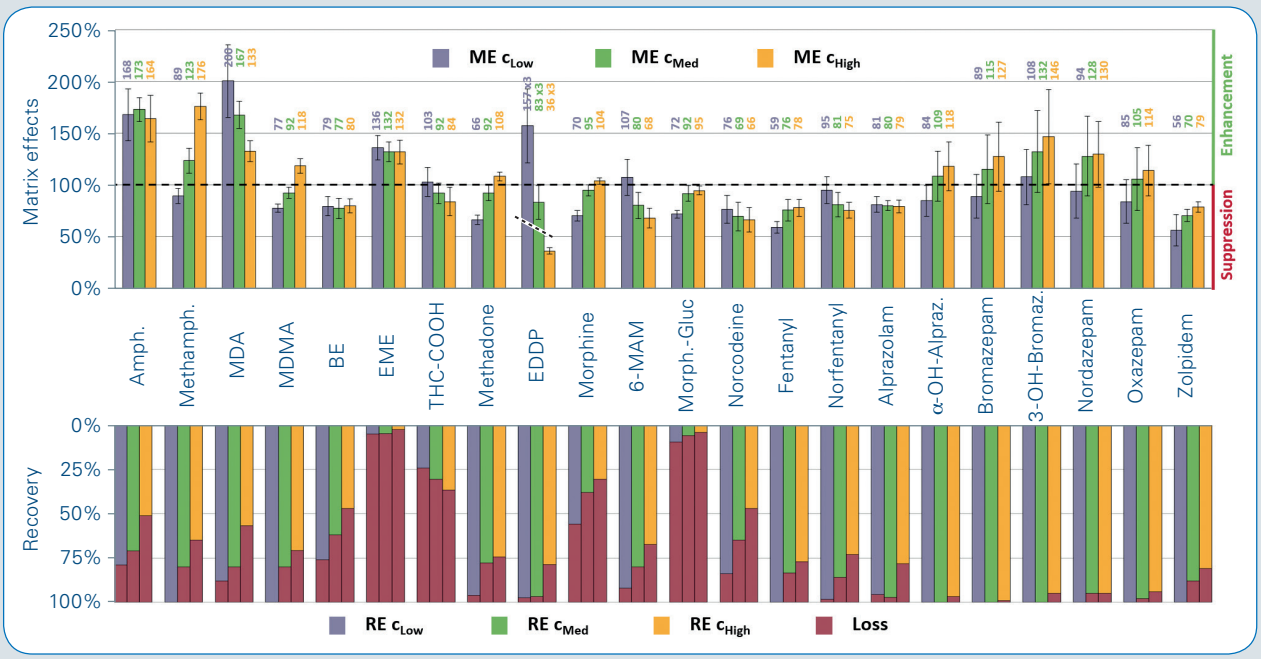
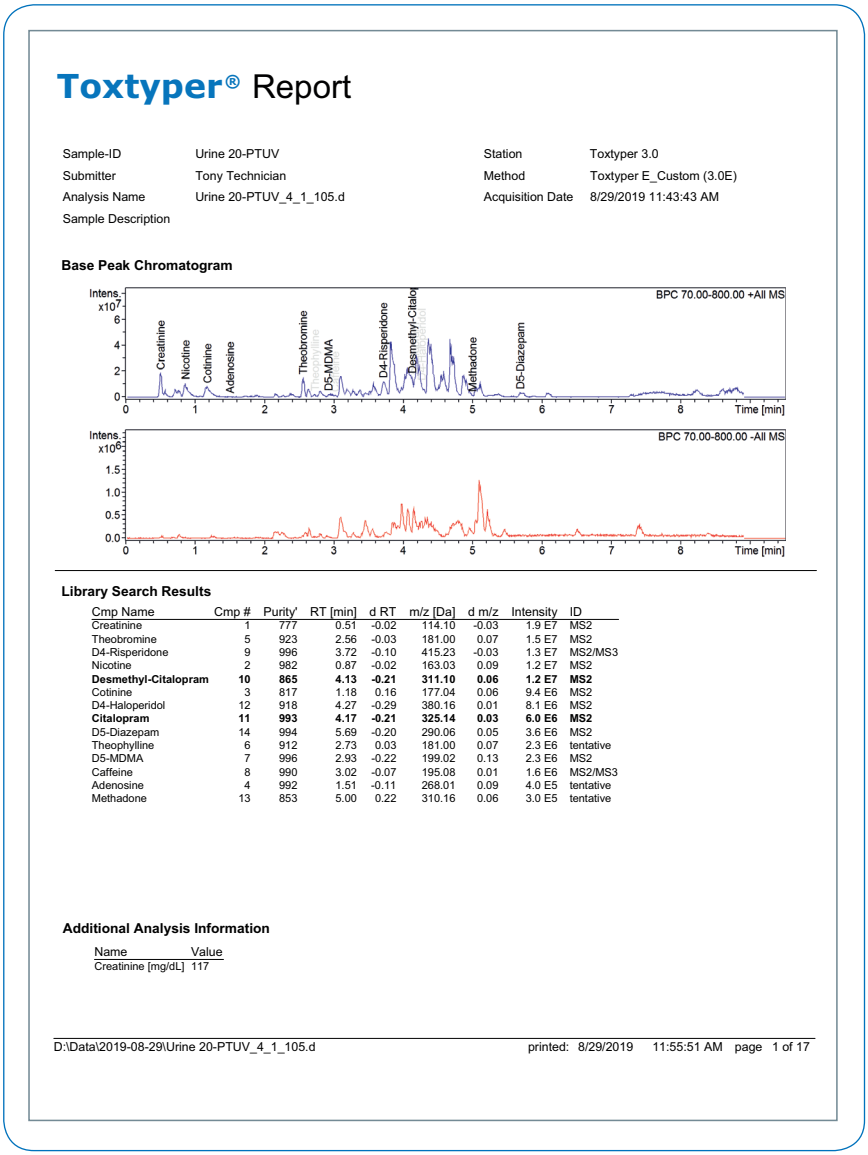


Figure 3: Average ME and RE for 22 selected analytes spiked into six different urines at three concentrations. Samples were analyzed in duplicate.



Toxtyper® Report

Sample-ID	Urine 20-PTUV	Station	Toxtyper 3.0
Submitter	Tony Technician	Method	Toxtyper E_Custom (3.0E)
Analysis Name	Urine 20-PTUV_4_1_105.d	Acquisition Date	8/29/2019 11:43:43 AM
Sample Description			

Base Peak Chromatogram

Library Search Results

Cmp Name	Cmp #	Purity'	RT [min]	d RT	m/z [Da]	d m/z	Intensity	ID
Creatinine	1	777	0.51	-0.02	114.10	-0.03	1.9 E7	MS2
Theobromine	5	923	2.56	-0.03	181.00	0.07	1.5 E7	MS2
D4-Risperidone	9	996	3.72	-0.10	415.23	-0.03	1.3 E7	MS2/MS3
Nicotine	2	982	0.87	-0.02	163.03	0.09	1.2 E7	MS2
Desmethyl-Citalopram	**10**	**865**	**4.13**	**-0.21**	**311.10**	**0.06**	**1.2 E7**	**MS2**
Cotinine	3	817	1.18	0.16	177.04	0.06	9.4 E6	MS2
D4-Haloperidol	12	918	4.27	-0.29	380.16	0.01	8.1 E6	MS2
Citalopram	**11**	**993**	**4.17**	**-0.21**	**325.14**	**0.03**	**6.0 E6**	**MS2**
D5-Diazepam	14	994	5.69	-0.20	290.06	0.05	3.6 E6	MS2
Theophylline	6	912	2.73	0.03	181.00	0.07	2.3 E6	tentative
D5-MDMA	7	996	2.93	-0.22	199.02	0.13	2.3 E6	MS2
Caffeine	8	990	3.02	-0.07	195.08	0.01	1.6 E6	MS2/MS3
Adenosine	4	992	1.51	-0.11	268.01	0.09	4.0 E5	tentative
Methadone	13	853	5.00	0.22	310.16	0.06	3.0 E5	tentative

Additional Analysis Information

Name	Value
Creatinine [mg/dL]	117

D:\Data\2019-08-29\Urine 20-PTUV_4_1_105.d printed: 8/29/2019 11:55:51 AM page 1 of 17

The Toxtyper® library includes many endogenous and common compounds (e.g., creatine, cortisone, ibuprofen, among many others), adding depth and validity to true toxicology screens. In the example report shown in Figure 4, caffeine and two metabolites (theobromine and theophylline) were detected, along with nicotine and its metabolite cotinine.

As shown, the complete automation of the Toxtyper® workflow - from the sample preparation to the final report - makes it a robust and effective tool within systematic toxicological analysis (STA). Further, the overall speed and operational ease of the workflow is well-suited to support routine screening and 24/7 emergency toxicology requirements.

Figure 4: Toxtyper® report title page from an archived urine sample screened via online µSPE-LC-MS^n indicating the presence of both the antidepressant citalopram and its metabolite desmethyl-citalopram.

Conclusion

- By implementing an online µSPE into the Toxtyper™ workflow, a fully automated urine screening from sample preparation to final report has been achieved.
- Overall, LOD values using online µSPE are comparable or superior to traditional protein precipitation for the tested compounds. In the reanalysis of true toxicology samples, online µSPE was in good agreement with the findings from routine analysis, and even allowed the detection of both parent and metabolites for some of the tested compounds.
- As an alternative or companion to immunoassays, the use of online µSPE with an LC-MS^n workflow enables rapid, reproducible, and unambiguous detection of a wide array of drug compounds.
- This complete analytical solution minimizes inter-individual differences in sample handling, increasing result repeatability (both within and between laboratories) and improving laboratory operating efficiency.

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