The missing step towards fully automated LC-MS screening of urine samples - Implementation of µSPE into the analytical workflow

Michaela Schmidt[1,2], Marina Schumacher[3], Birgit Schneider[3], Laura M. Huppertz[2], Jürgen Kempf[2]

[1]*Faculty Medical and Life Sciences, Furtwangen University, Schwenningen, Germany;*
[2]*Institute of Forensic Medicine, Forensic Toxicology, Medical Center - University of Freiburg, Germany;* [3]*Bruker Daltonik GmbH, Bremen*

Institute of Forensic Medicine
Forensic Toxicology

Introduction

Systematic toxicological analysis (STA) using comprehensive screening approaches is a major part of everyday work in forensic toxicology, usually involving different analytical techniques. The great benefit of immunoassays (IA) in routine screening of body fluids is the high degree of automation regarding sample preparation and reporting of results.

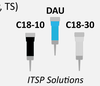
During the last decade, LC-MS has become a key technique in STA, but in contrast to IA an appropriate sample preparation is crucial for screening of body fluids. Offline liquid-liquid extraction (LLE), solid phase extraction (SPE) or protein precipitation (PP) are often laborious but mandatory steps and their integration into the analytical workflow is the missing piece towards a fully automated routine LC-MS analysis.

The aim of this project was to implement an online µSPE into an existing LC-MS method to achieve a fully automated LC-MS screening of urine samples.

Method Development

Starting Equipment

- Routine LC-MS^n screening (Toxtyper®, TT)
- LC-QTOF-MS screening (TargetScreener, TS)
- Elute UHPLC equipped with a PAL RTC
- Three types of smartSPE^{TM} cartridges:
  - UCT C18 endcapped cartridges 10 mg
  - UCT DAU cartridges 10 mg
  - UCT C18 endcapped cartridges 30 mg



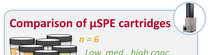
General Proof of Concept

Low, med., high concentration

Comparison of µSPE and routine PP

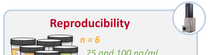
Comparison of µSPE cartridges

n = 6
Low, med., high conc.



Reproducibility

n = 6
25 and 100 ng/ml



Recovery and Matrix Effects

27.5, 275 and 500 ng/ml

Limit of Detection

25, 50, 75 and 100 ng/ml

Real Samples

n = 50

Comparison of µSPE and PP using urine from routine cases (post / ante mortem)

Methods

PAL RTC µSPE System and Elute UHPLC

µSPE Steps

1. Washing:	0.2% NH_4 acetate in methanol
2. Conditioning:	20% NH_4 acetate in H_2O
3. Sample Load:	200 µl urine
4. Desalting:	20% NH_4 acetate in H_2O
5. Elution:	0.2% NH_4 acetate (V = 75 µl)

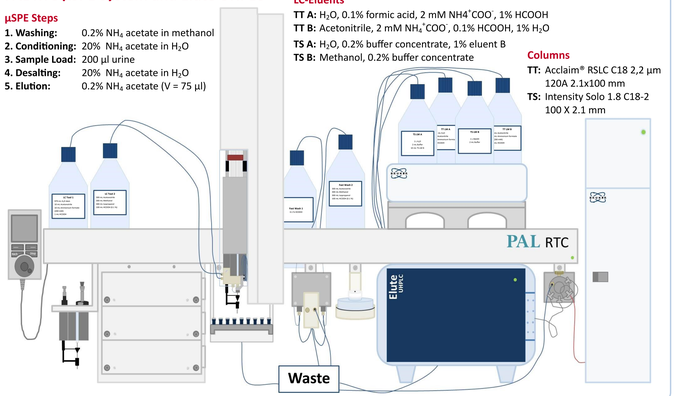
LC-Eluents

TT A: H_2O, 0.1% formic acid, 2 mM $NH_4^+COO^-$, 1% HCOOH
TT B: Acetonitrile, 2 mM $NH_4^+COO^-$, 0.1% HCOOH, 1% H_2O

TS A: H_2O, 0.2% buffer concentrate, 1% eluent B
TS B: Methanol, 0.2% buffer concentrate

Columns

TT: Acclaim® RSLC C18 2,2 µm 120A 2.1x100 mm
TS: Intensity Solo 1.8 C18-2 100 X 2.1 mm



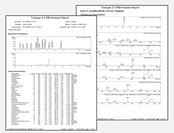
Bruker amaZon Speed ion trap

ESI (zero delay polarity switching)
UltraScan: 70 - 800 Da (32.000 Da/s)
AutoMSn mode up to MS³
Scheduled Precursor list with 1000 comp.

Bruker impact II QTOF

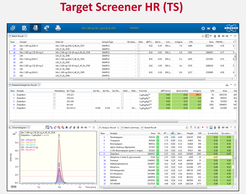
ESI positive mode
Scan Range: 30 - 1000 Da
Full Scan TOF-MS / bbCID (alternating)

Toxtyper Screening (TT)



Automated data evaluation and reporting of results

Target Screener HR (TS)



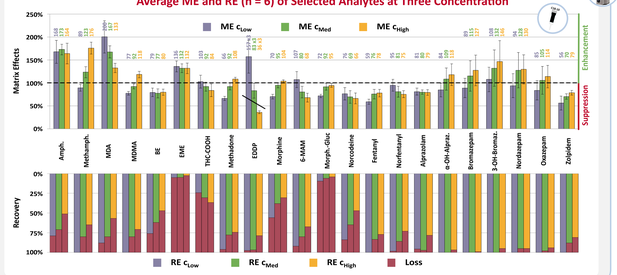
Automated evaluation of HR data using a database of over 1700 drugs, drugs of abuse and metabolites

Matrix Effects and Recovery

As ion source and ion transfer of both instruments are identical, ME and RE were only evaluated by QTOF-MS using a protocol adapted from Matuszewski et al.. For the C18-10 cartridge, maximum ion suppression in six tested urine matrices was around 50%. DAU cartridges showed comparable matrix effects in a pooled urine matrix. While ion suppression will have negative effects on the LOD, ion enhancement is not an issue in screening approaches.

For morphine-glucuronide and EME the overall recovery was very poor. The C18-10 material seems to have problems properly retaining these early eluting compounds. Average RE and ME of tested analytes are depicted on the right hand side.

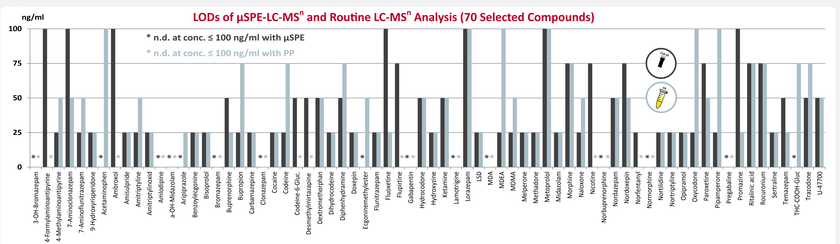
Average ME and RE (n = 6) of Selected Analytes at Three Concentration



Limits of Detection (LOD)

LOD for the µSPE-LC-MS^n screening approach were determined in pooled blank urine (n = 10) fortified with compounds most often found in routine case work of the last year, in decreasing concentrations down to 25 ng/ml. The lowest concentration automatically identified (n = 3) was set as LOD.
These LODs are suitable for STA in emergency and post mortem toxicology, but not sufficient for trace analysis as required for sobriety testing or analysis of DFC cases.

LODs of µSPE-LC-MS^n and Routine LC-MS^n Analysis (70 Selected Compounds)



Conclusions

- The presented workflow is the first Toxtyper / TargetScreener approach featuring complete automation from sample preparation to evaluation of data. The extraction time of about 14 min fits into the runtime of the QTOF-Screening (20 min) and only slightly exceeds the Toxtyper runtime (11 min).
- LC-MSn screening of fortified urine using µSPE led to similar or better results than the routine sample preparation. This results could be confirmed in a batch of urine samples from real cases. All µSPE-LC-MSn screening results were in good agreement with the initial routine analysis.
- LODs of the QTOF are probably lower than the ones found for the Toxtyper and will be determined separately.
- Evaluation of ME showed a maximum ion suppression of 50% which was considered acceptable for a screening approach.
- Implementation of a cleavage step would overcome the extraction issues seen for some of the glucuronides and therefore might lower the LOD of the respective parent substance but would also prolong the overall extraction time.
- Direct injection of the µSPE eluate limits the choice of solvents that could be used for extraction. Optimization of the protocol, including incorporation of an evaporation unit, might increase the overall performance of the DAU cartridge.

Results

General Proof of Concept

In comparison to routine PP with acetonitrile, the identification rate of the LC-MS^n screening could be improved from 74% to 84% at low concentration levels and from 90% to 96% at high concentration levels, when using µSPE.

Due to the higher sensitivity of the QTOF-MS system all spiked compounds could be detected even at low concentrations.

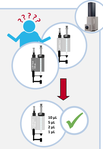
Comparison of µSPE Cartridges

18 compounds of different compound classes covering the retention time and mass range of the method was chosen for further testing of different µSPE cartridges. For six analytes, higher S/N ratios could be observed using the DAU cartridges. For all other analytes, no preferable cartridge could be determined. C18-30 cartridges led to higher S/N ratios for EME and norbuprenorphine but to low absolute peak areas, probably due to higher amounts of sorbent. Higher eluent volumes might enhance the elution but would also dilute the extract injected to the LC-MS system. Therefore, the C18-30 cartridge was excluded from further method development.

Reproducibility

First tests using two different PAL tools for sample and solvent handling - to circumvent carry over at all costs - led to poor results concerning reproducibility. So, a new 250 µl LC-MS Tool was tested for all liquid handling including the injection step. Injection reproducibility of different volumes (1, 2, 5 and 10 µl) ranged from 1.5 to 7%. Optimizing the cleaning procedures after the different extraction steps led to no detectable carryover caused by the µSPE system.

Reproducibility of the complete extraction process using a single tool was tested by tenfold preparation of pooled urine fortified with chosen compounds. Morphine-glucuronide was the only outlier in this test with RSD_{Low} of 23.3 and 19.8% and RSD_{High} of 75.7 and 38.3%.



RSD of ten fold extraction

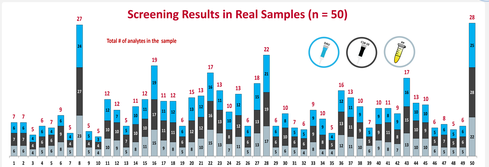
	C18-10	DAU
Low	5.6 - 10.9%	10.0 - 14.9%
High	4.3 - 11.2%	1.8 - 6.8%

Real Samples

The µSPE-LC-MS^n screening results of 28 urine and 22 post-mortem urine samples from real cases were in good agreement with the findings from routine analysis. Using µSPE, 90% (C18-10) and 88% (DAU) of the substances could be identified in accordance to routine analysis. Routine LC-MS^n screening could identify 80% of the compounds. The sum of all different analytes identified by µSPE and PP and confirmed by further routine analysis corresponds to 100%.

Unfortunately, neither of the cartridges could extract ethyl glucuronide (EtG) and ethyl sulfate (EtS) which were therefore excluded from this evaluation.

Screening Results in Real Samples (n = 50)



In one post-mortem case, the antiparkinson drug pramipexole could be detected in both µSPE runs but not in the initial routine screening. The routine Toxtyper only identified pramipexole in the corresponding cardiac blood and vitreous humor. In a second case, amphetamine could be found in both µSPE runs but not in the routine Toxtyper. The amphetamine finding was confirmed by the more sensitive LC-QTOF-MS.