Implementation of an Online µSPE - The Final Step Towards Fully Automated LC-MS Urine Screening in Forensic Toxicology

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Introduction

Systematic toxicological analysis (STA) is a major part of everyday work in forensic toxicology. Immunological screening offers great advantages in automation of sample preparation and reporting of results but neither the quantitative nor the qualitative information from immunoassays is admissible in court.

During the last decade, LC-MS has become a key technique in STA, but in contrast to immunoassays 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 to an existing LC-MS method to achieve a fully automated LC-MS screening of urine samples.

Results

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.

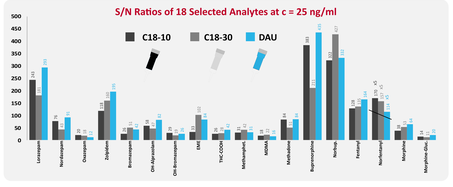
Due to higher sensitivity of the QTOF-MS system all spiked compounds could be detected even at low concentrations.

Comparison of Cartridges

For further testing, 18 compounds covering different compound classes, as well as retention time and mass range of the method were chosen.

For six analytes, higher S/N ratios could be observed using DAU cartridges. No preferable cartridge could be determined for all other analytes.

C18-30 cartridges led to higher S/N ratios for ecgonine methyl ester (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 subsequently injected to the LC-MS system. Therefore, the C18-30 cartridge was excluded from further method development.



Reproducibility

Reproducibility was tested by QTOF-MS and data evaluation was carried out by comparing peak area ratios ($area_{analyte}/area_{ISTD}$). First tests using two different PAL tools for sample and eluent 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 liquid handling including the injection. Injection reproducibility of different injection 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 a chosen set of 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 tenfold extraction

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

Matrix Effects and Recovery

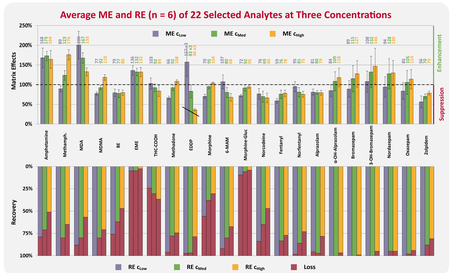
Matrix effects (ME) and recovery (RE) were evaluated by QTOF-MS using a protocol adapted from Matuszewski et al. For the C18-10 cartridge, maximum ion suppression in six tested urine matrices was around 50%. DAU cartridges showed comparable matrix effects in a pooled urine matrix. While ion suppression will have an effect on the limits of detection, ion enhancement is not an issue in screening approaches.

For morphine-glucuronide and ecgonine methyl ester (EME) the overall recovery was very poor. The C18-10 cartridge seems to have some problems retaining these early eluting compounds.

This could be improved by using the DAU cartridge, but still recovery of these compounds is not acceptable.

RE and ME in the different urine samples are shown in the table on the left. Average RE and ME of all tested analytes are depicted on the right.

ME (%)	Urine A	Urine B	Urine C	Urine D	Urine E	Urine F
c_{Low}	65 - 423	59 - 470	51 - 453	56 - 529	66 - 470	26 - 489
c_{Med}	76 - 261	63 - 241	64 - 258	76 - 252	75 - 267	46 - 222
c_{High}	74 - 168	69 - 183	66 - 200	75 - 157	57 - 183	52 - 199
RE (%)	**Urine A**	**Urine B**	**Urine C**	**Urine D**	**Urine E**	**Urine F**
c_{Low}	44 - 118	53 - 133	55 - 199	76 - 198	47 - 110	47 - 85
c_{Med}	53 - 132	32 - 121	33 - 110	43 - 133	27 - 99	39 - 208
c_{High}	24 - 99	35 - 116	27 - 96	38 - 114	31 - 148	29 - 75
Urea (mg/dL)	*30.7*	*70.1*	*186.4*	*31.9*	*112.6*	*131.2*



Methods

Starting Equipment

- Routine LC-MS^n (Toxtyper® (TT)) and LC-QTOF-MS (TargetScreener HR (TS)) screening
- Elute UHPLC system (Bruker Daltonik) with PAL RTC sampler (CTC)
- Three types of smartSPE™ cartridges (ITSP Solutions)

UCT C18 endcapped cartridges 10 mg (C18-10)
UCT DAU cartridges 10 mg (DAU)
UCT C18 endcapped cartridges 30 mg (C18-30)

LC-Eluents
TT A: 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: 0.1% 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

Method Development

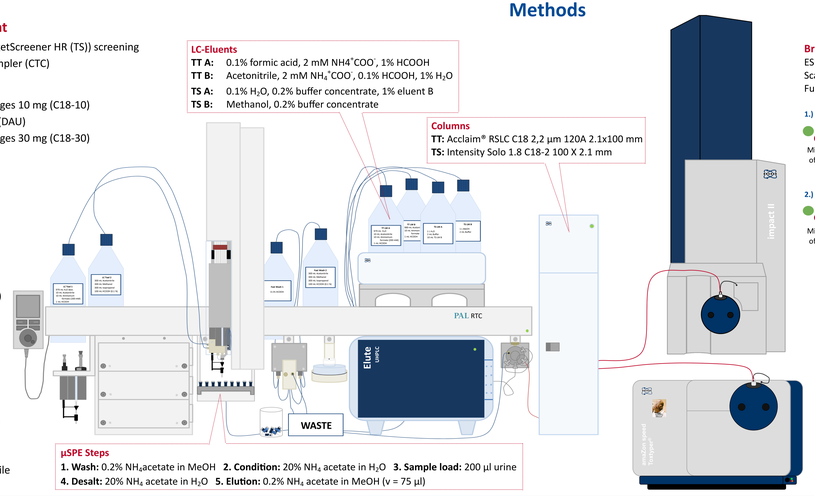
A) General Proof of Concept
- C18-10 cartridges
- Substances of forensic interest (n = 139)
- Low and high concentration

B) Comparison of µSPE Cartridges
- C18-10, C18-30 and DAU cartridges
- Low, med, high concentration in pooled urine (n = 6)
- Triplicates

C) Reproducibility
- C18-10 and DAU cartridges
- 25 ng/ml and 100 ng/ml in pooled urine (n = 6)
- Tenfold extraction

D) Matrix Effects, Recovery and Limits of Detection
- C18-10 cartridges
- ME and RE: 27.5, 275 and 550 ng/ml in urine (n = 6)
- Duplicates (ME and RE), triplicates (LOD)

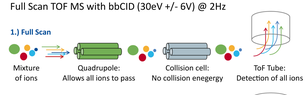
E) Real Samples
- µSPE (C18-10 and DAU) vs routine PP with acetonitrile
- n = 50 (ante mortem / post mortem)

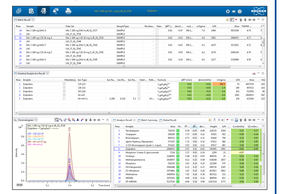


µSPE Steps
1. Wash: 0.2% NH_4acetate in MeOH **2. Condition:** 20% NH_4 acetate in H_2O **3. Sample load:** 200 µl urine
4. Desalt: 20% NH_4 acetate in H_2O **5. Elution:** 0.2% NH_4 acetate in MeOH (v = 75 µl)

TargetScreener HR QTOF-Screening (TS)

Bruker impact II QTOF
ESI positive mode
Scan range: 30 - 1000 Da
Full Scan TOF MS with bbCID (30eV +/- 6V) @ 2Hz

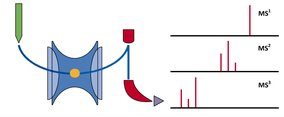


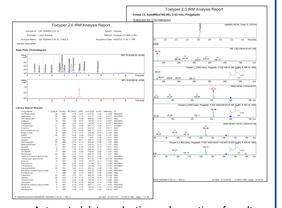


Automated evaluation of HR data using a database containing over 1700 drugs, drugs of abuse and metabolites

Toxtyper Screening (TT)

Bruker amaZon Speed
ESI (zero delay polarity switching)
AutoMSn mode: 70 - 800 Da (32.000 Da/s) up to MS^3
Scheduled Precursor list with 1000 compounds

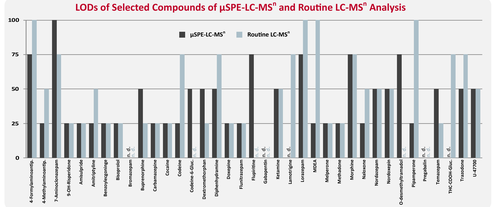




Automated data evaluation and reporting of results

Limits of Detection (LOD)

Limits of detection for the µSPE-LC-MS^n screening approach were determined in pooled blank urine (n = 10) fortified with different mixtures of drugs and drugs of abuse in decreasing concentrations down to 25 ng/ml. Compounds found most in routine cases of the last year were chosen for this evaluation and the lowest concentration automatically identified (n = 3) was set as LOD.



Analysis of Real Samples

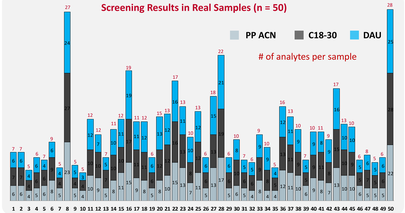
The µSPE-LC-MS^n screening results of 28 urine and 22 post-mortem urine samples from real cases were in good agreement with the findings from routine analysis.

Using µSPE, 90 % (C18-10) and 88 % (DAU) of the substances could be identified in accordance to routine analysis. Routine LC-MS^n screening with PP ACN could identify 80 % of the compounds. The sum of all different analytes identified by µSPE and PP corresponds to 100 %.

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

In one case, the antiparkinson drug pramipexole could be detected in both µSPE runs but not in the routine urine screening. The 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 LC-QTOF-MS.



Conclusion

- The chosen hardware can be implemented in both routine workflows enabling a completely automated LC-MS screening approach from sample preparation to data evaluation.
- 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-MS^n 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 real urine samples. All µSPE-LC-MS^n screening results were in good agreement with the initial routine analysis.
- The alcohol consumption markers EtG and EtS could not be detected with neither of the tested cartridges.
- Both cartridges showed satisfactory recoveries for screening, except for EME and morphine-glucuronide.
- Evaluation of matrix effects showed a maximum ion suppression of 50% which was considered sufficient for a screening approach.
- The µSPE-LC-MS^n method showed LODs comparable to the actual routine approach or slightly better. Of course, the LODs highly depend on the sensitivity of the used MS system.
- Direct injection of the µSPE eluate limits the choice of solvents that could be used for extraction. Further optimization of the protocol might increase the overall performance of the DAU cartridge.