The final step towards automated LC-MS screening - Implementation of an online µSPE for urine screening

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Introduction

The great benefit of immunoassays in routine screening of body fluids is the high degree of automation regarding sample preparation and reporting of results. In contrast, an appropriate sample preparation is crucial for LC-MS analysis 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 fully automated routine LC-MS screening.
The aim of this project was to implement an online µSPE to achieve a fully automated LC-MS screening approach for urine samples in forensic toxicology, including examination of different cartridges, optimization of extraction steps, reproducibility, determination of analyte recovery and preliminary data on the analysis of real samples.

Methods

Starting Equipment

- Routine LC-MS^n (Toxtyper®, TT) and LC-qToF-MS (TargetScreener, TS) screening approach
- Elute UHPLC (Bruker) equipped with a PAL RTC (CTC)
- Three types of smartSPE™ cartridges (ITSP Solutions)

UCT C18 endcapped cartridges 10 mg
UCT DAU cartridges 10 mg
UCT C18 endcapped cartridges 30 mg

Method Development

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

B) Comparison of Cartridges
- C18-10, C18-30, DAU
- Low, med, high conc. in pooled urine (n = 6)
- Triplicates

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

D) Recovery and Matrix Effects
- C18-10 and DAU
- c = 27.5, 55, 110 ng/ml in pooled urine (n = 6)
- Duplicates

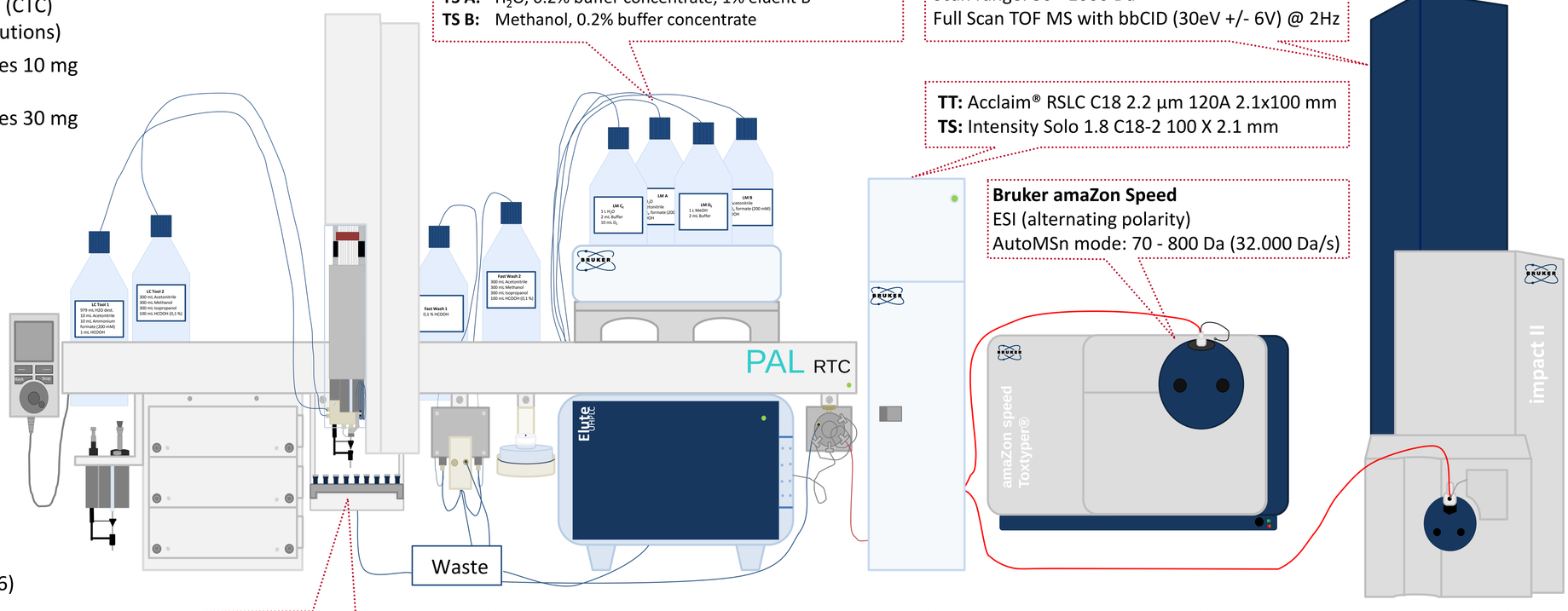
E) Real Samples
- µSPE (C18-10, DAU) vs routine PP ACN
- n = 12 (ante mortem / post mortem)

TT A: H_2O, 0.1% HCOOH, 2 mM $NH4^+COO^-$, 1% acetonitrile
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

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

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
ESI (alternating polarity)
AutoMSn mode: 70 - 800 Da (32.000 Da/s)



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

Analytical Results

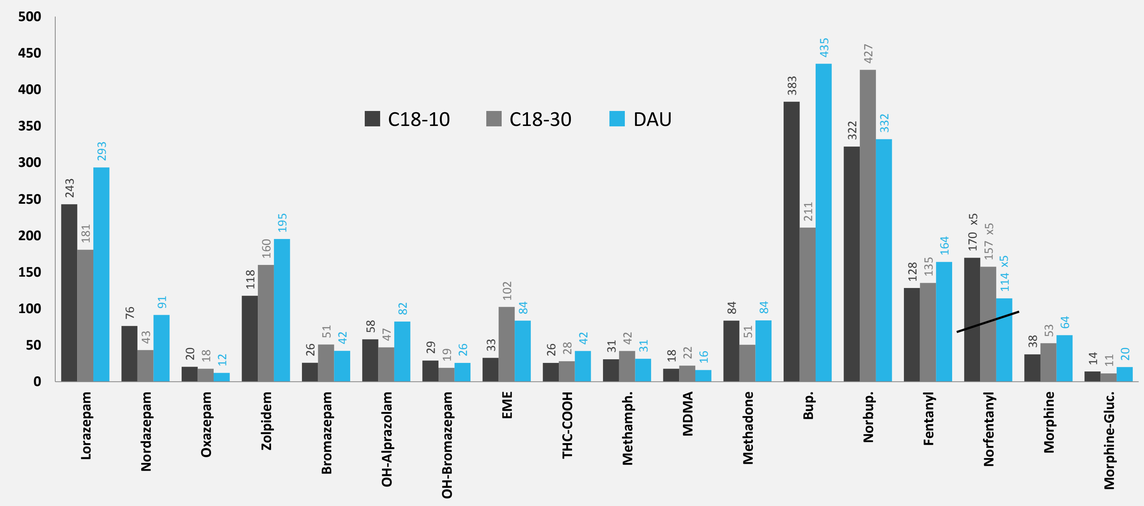
Proof of Concept

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, respectively, 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, a set of 18 compounds of different compound classes covering the retention time and mass range of the method was chosen.
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.

S/N Ratios of 18 Selected Analytes at c = 25 ng/ml



Reproducibility

Analysis was carried out by qToF-MS and data evaluation 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%. Adjusting the cleaning procedures after the different extraction steps led to no detectable carryover caused by the µSPE system.

RSD of ten fold 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%

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. RSDs of the peak area ratios are shown on the left. 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%.

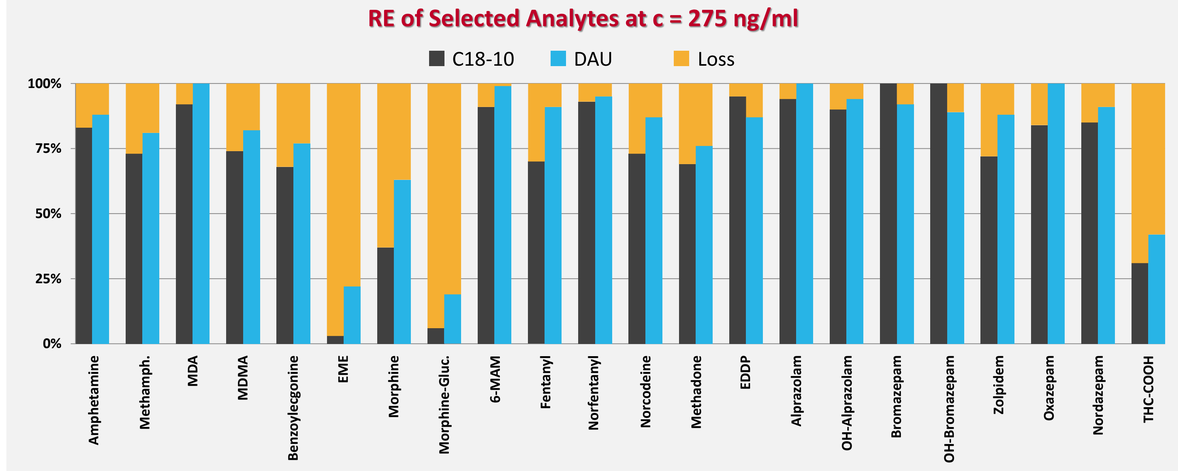
Recovery and Matrix Effects

Recovery (RE) and matrix effects (ME) were evaluated using a protocol adapted from Matuszewski et al.
For morphine-glucuronide and EME the overall recovery was very poor. The C18-10 cartridge seems to have severe problems retaining these early eluting compounds.

	RE		ME	
	C18-10	**DAU**	**C18-10**	**DAU**
Low	50 to 90%	61 to 100%	53 - 227%	52 - 291%
Med	37 to 100%	63 to 100%	56 - 202%	53 - 205%
High	32 to 93%	53 to 91%	57 - 164%	61 - 184%

This could be improved by using the DAU cartridge, but still recovery of these two compounds is not acceptable. Recovery of all other tested analytes are shown in the table above.
Both cartridges showed comparable matrix effects. Maximum ion suppression in pooled matrix was around 50%. This test will be rerun using the appropriate number of different matrices with the conclusive cartridge.

RE of Selected Analytes at c = 275 ng/ml



Real Samples

Preliminary data of real urine samples (ante and post mortem) was in good agreement with the findings of the routine LC-MS^n screening approach. In one case, extraction using the DAU cartridge missed naloxone.
Unfortunately, neither of the cartridges could extract the alcohol consumption markers ethyl glucuronide and ethyl sulfate.
In one post mortem urine, 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 neither in the routine Toxtyper, nor in the additional MWW screening. The amphetamine finding was subsequently confirmed by LC-qToF-MS.

Post Mortem Urine

ACN PP	C18-10	DAU
-	Pramipexole	Pramipexole
Morphine	Morphine	Morphine
Morphine-3-gluc.	Morphine-3-gluc.	Morphine-3-gluc.
Morphine-6-gluc.	Morphine-6-gluc.	Morphine-6-gluc.
Ketamine	Ketamine	Ketamine
Norketamine	Norketamine	Norketamine
9-OH-Risperidone	9-OH-Risperidone	9-OH-Risperidone
Quetiapine	Quetiapine	Quetiapine
Norquetiapine	Norquetiapine	Norquetiapine
Haloperidol	Haloperidol	Haloperidol
Cortisone	Cortisone	Cortisone

Conclusion

- The chosen hardware can be implemented in both routine workflows enabling a completely automated LC-MS screening approach 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-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 small batch of real urine samples. All µSPE-LC-MS^n screening results were in good agreement with the initial routine screening.
- Ethyl glucuronide and ethyl sulfate could not be extracted with neither of the tested cartridges.
- The new 250 µl LC-MS Tool of the PAL RTC showed no detectable carryover.
- Both cartridges showed satisfactory recoveries for screening, except for EME and morphine-glucuronide.
- Preliminary evaluation of matrix effects showed a maximum ion suppression of 50%.
- 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.
- LODs depend on the sensitivity of the used MS system and have to be determined separately.