Federal public Service
Justice

High Throughput Analysis of Amphetamines in Urine with On-line-Solid Phase Extraction-Liquid-Chromatography-Tandem Mass Spectrometry

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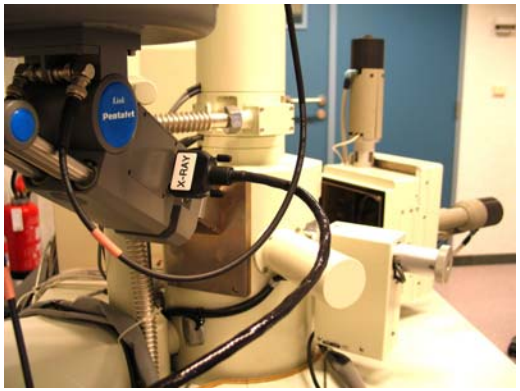
.be

Introduction

- The NICC is part of the Ministry of Justice and carries out technical and scientific research for the purpose of crime solving.

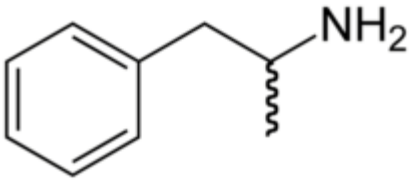
- **Missions**
  - to carry out forensic casework
  - to conduct Research & Development
  - to act as a centre of forensic knowledge and expertise

- **The NICC provides forensic expertise in the following areas:**
  - **Toxicology**
  - DNA-analysis
  - Forensic investigation of hair, fibres and textile
  - Investigation of weapons & ammunition
  - Gunshot residue investigation
  - Explosives
  - Forensic investigation of fire
  - Drug analysis
  - General Chemistry (e.g. fire-accelerators and paint)



Introduction

- stimulants or "uppers"
- usually made synthetically in illegal labs.
- stimulation of the central nervous system, a sense of well-being and higher energy, a release of social inhibitions, and feelings of cleverness, competence and power.



Amphetamine

CLANDESTINE METHAMPHETAMINE LAB

Introduction

- the number of illegal users of amphetamines increases dramatically, the determination of these drugs has become an important task

Urine sample analysis is generally used to examine the abuse of these stimulants

Introduction

As a routine urinalysis laboratory, there is an increasing demand for an accurate and fast analysis in a cost-effective way .

LC-MS has emerged as a sensitive and selective analytical method in drug analysis.

A sample pre-treatment is often needed to remove protein and potential interferences prior LC-MS analysis

Solid Phase Extraction (SPE) has been demonstrated as an effective sample pre-treatment procedure

Manual operations associated with theses process are very labor intensive and time-consuming

A lot of interest in recent years the direct injection of urine using an **on-line extraction method**

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A lot of interest in recent years the direct injection of urine using an **on-line extraction method**

The sample preparation step is embedded into the chromatographic separation

Introduction

On-line SPE Symbiosis Pharma System + Quattro Premier (Waters) MS/MS a fully integrated system

The autosampler can accommodate 24 96-well blocks

SPE Symbiosis Pharma System (Spark Holland)
+
Quattro Premier MS/MS (Waters)

SPE extraction unit can adapt 12 x 96-cartridges plates

A fully automated and unattended analysis of 1152 samples assuming the single use of each cartridge

After conditioning of the cartridge, loading of the sample and washing, and during the elution step, a second sample is loaded to an on-line SPE cartridge for the next analysis

The sample analysis cycle time approximates the LC run time without the time required for the SPE procedures

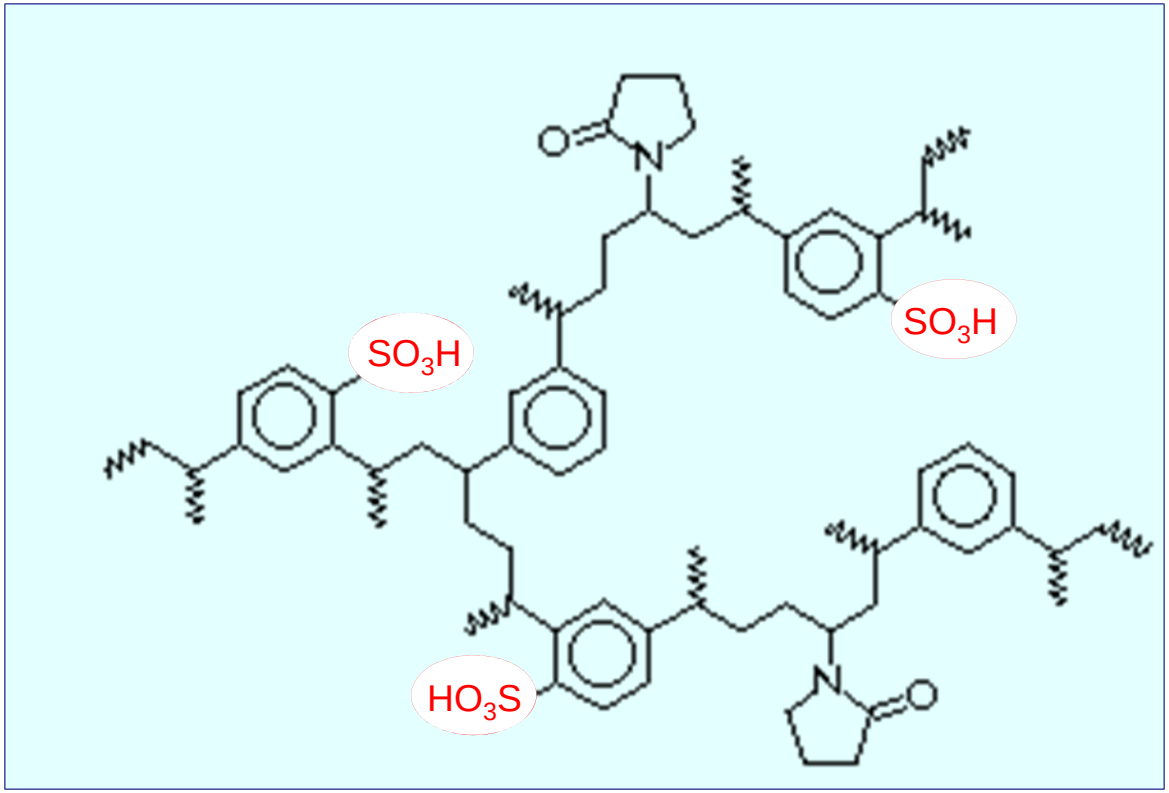
Introduction

Amphetamine
Methamphetamine
MDA
MDMA
MDEA
Ephedrine
PMA

IS

Amphetamine-d_{11}
Methamphetamine-d_5
MDA-d_5
MDMA-d_5
MDEA-d_6

SPE-LC-MS/MS



Oasis MCX cartriges (Waters)

Conditioning

2 mL Methanol,
5 mLmin

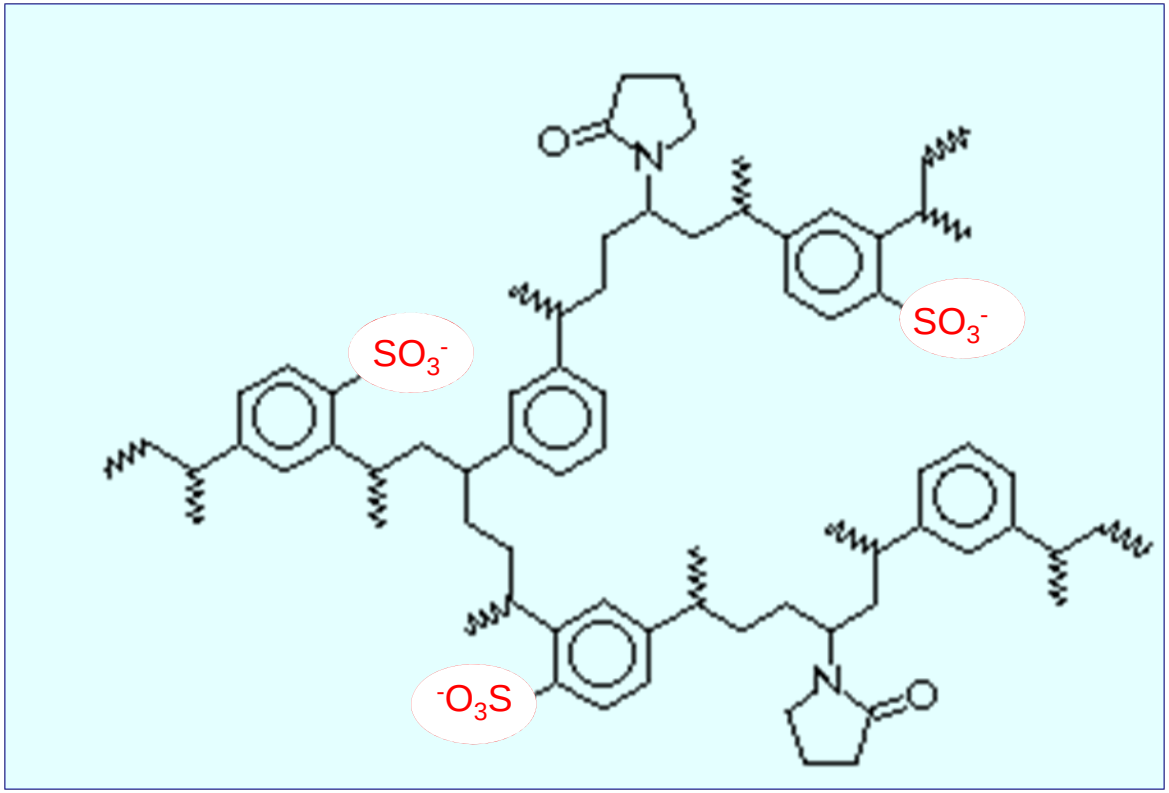
2 mL Methanol (5%NH3)
5 mLmin

2 mL Methanol,
5 mLmin

2 mL Methanol (5%NH3)
5 mLmin

- **Conditioning**
- **Equilibration**
- **Sample extraction**
- **Wash**
- **Elution**

SPE-LC-MS/MS



Oasis MCX cartriges (Waters)

Conditioning

↓

Equilibration

Sample extraction

Wash

Elution

Equilibration

2 mL Methanol, 0.1%FA
5 mLmin

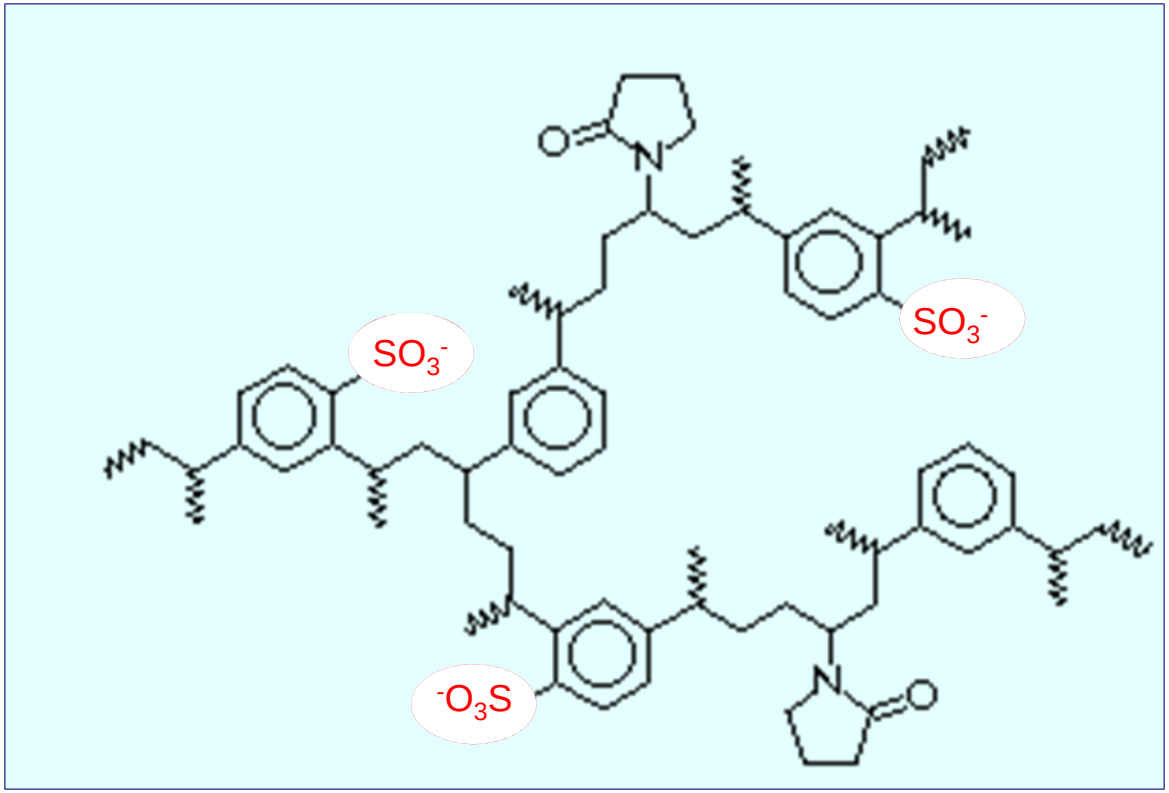
2 mL Methanol
5 mLmin

2 mL Methanol, 0.1% FA
5 mLmin

2 mL Water
5 mLmin

2 mL ACN, Water, 0.1%FA
5 mLmin

SPE-LC-MS/MS



Oasis MCX cartriges (Waters)

Conditioning

↓

Equilibration

Sample extraction

Wash

Elution

All these washes to reduce carryover!

Equilibration

2 mL Methanol, 0.1%FA
5 mLmin

2 mL Methanol
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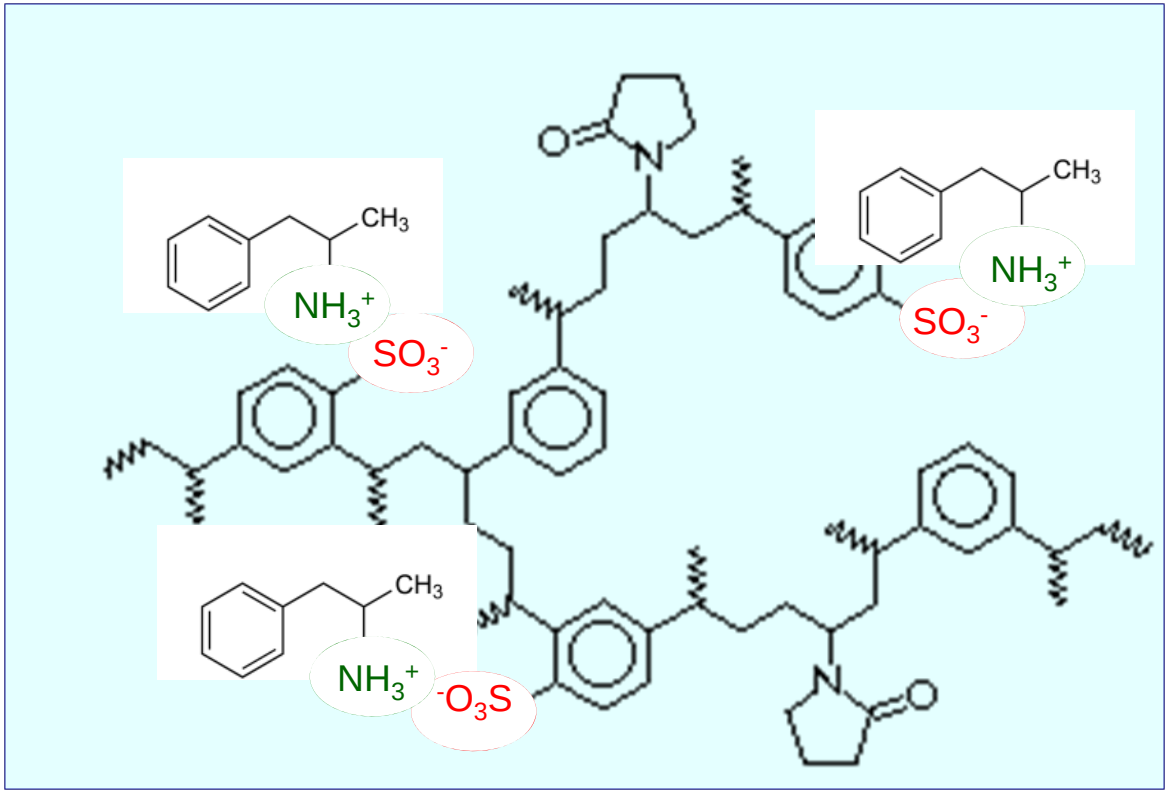
2 mL Methanol, 0.1% FA
5 mLmin

2 mL Water
5 mLmin

2 mL ACN, Water, 0.1%FA
5 mLmin

SPE-LC-MS/MS



Sample extraction

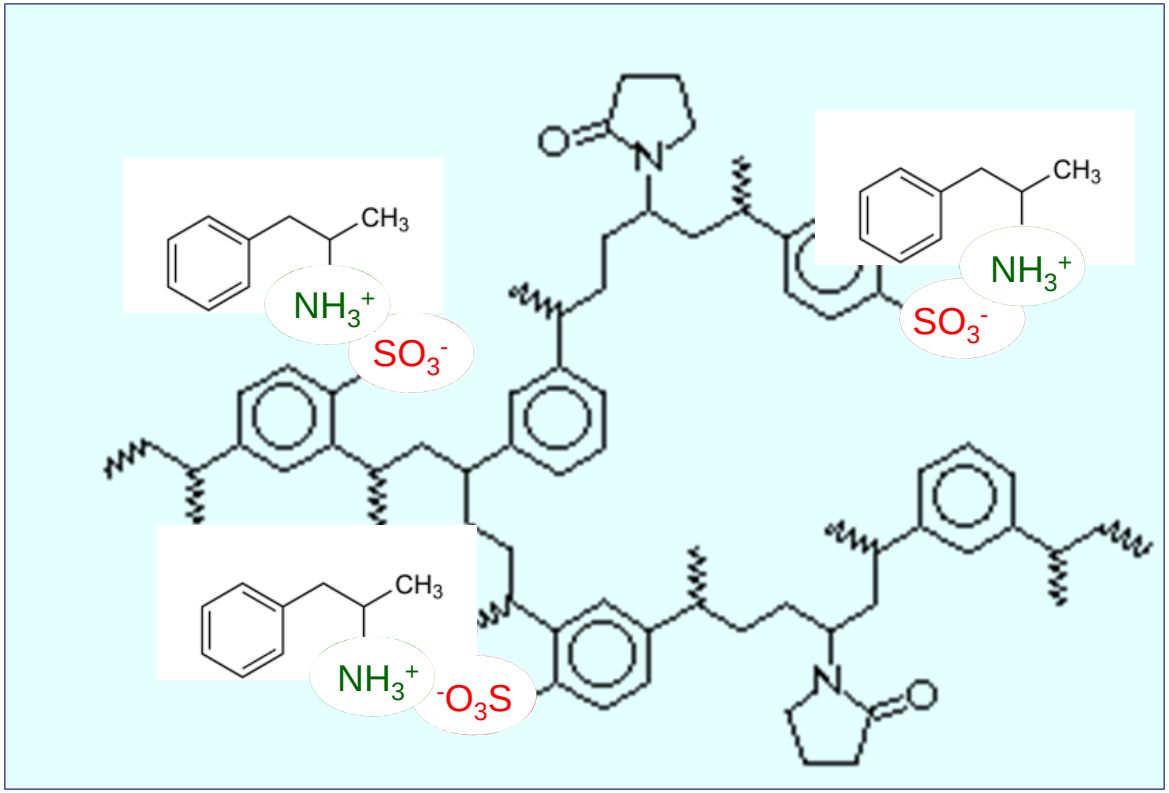
1 mL ACN, Water, 0.1%FA
0.5 mLmin

Oasis MCX cartriges (Waters)

- **Conditioning**
- **Equilibration**
- **Sample extraction**
- **Wash**
- **Elution**

Injection 30 µL (**50 µL urine** in 800 µL water 0.1%FA + IS)

SPE-LC-MS/MS



Oasis MCX cartriges (Waters)

Sample washes

2 mL ACN, Water, 0.1%FA
0.5 mLmin

2 mL Methanol, 0.1%FA
0.5 mLmin

- **Conditioning**
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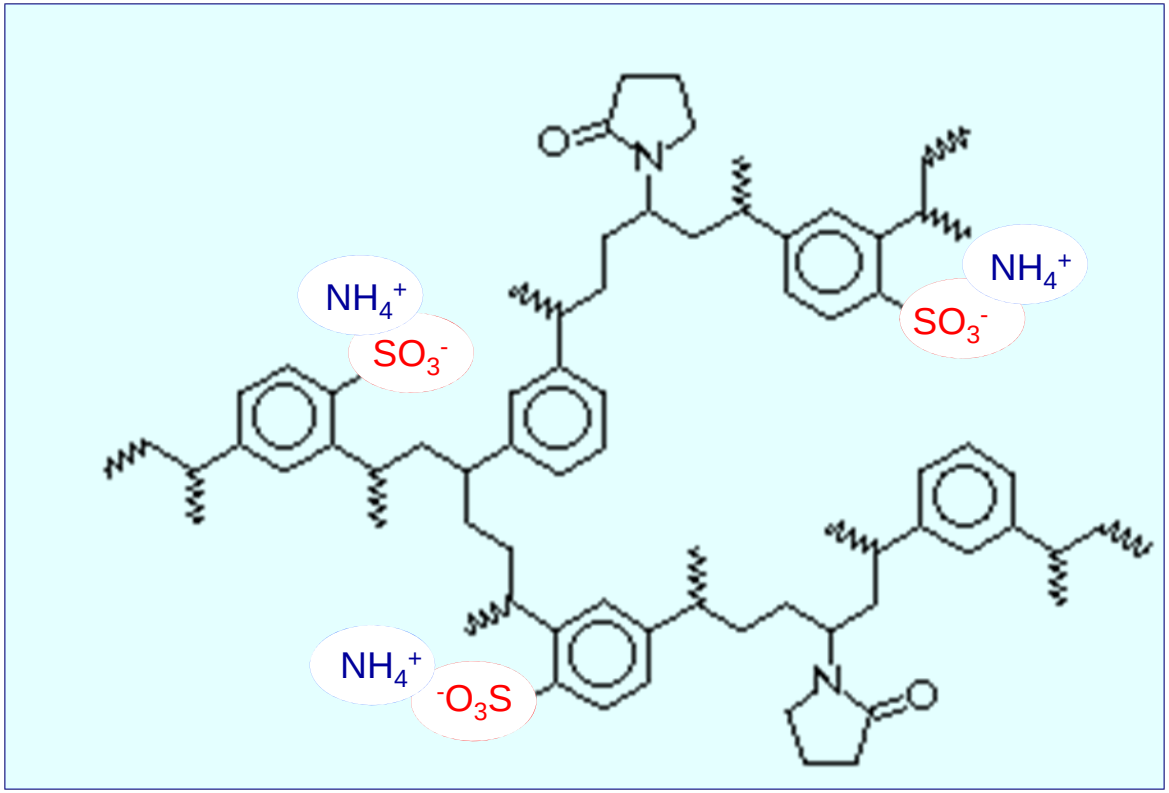
- **Elution**

SPE-LC-MS/MS



Elution

1 mL Methanol, 5% NH3
0.1 mLmin

Oasis MCX cartriges (Waters)

- **Conditioning**
- **Equilibration**
- **Sample extraction**
- **Wash**
- **Elution**

*While the first sample is eluted and analysed in the right clamp, a second sample is washed in paralel in the left clamp

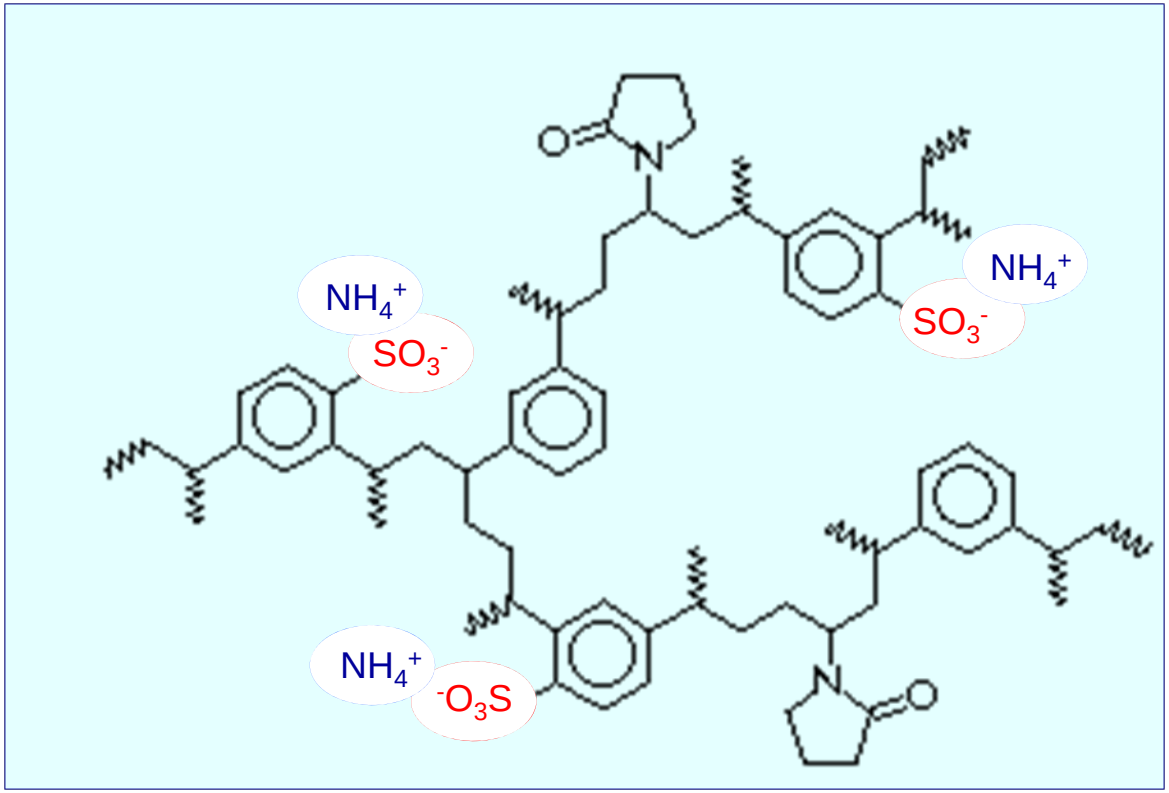
2 mL Methanol, 5 mLmin

2 mL Methanol (5%NH3) 5 mLmin

2 mL Methanol, 5 mLmin

⋮

SPE-LC-MS/MS



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2 mL Methanol, 5 mLmin

2 mL Methanol (5%NH3) 5 mLmin

2 mL Methanol, 5 mLmin

Several clamp, valve and syringe washes!

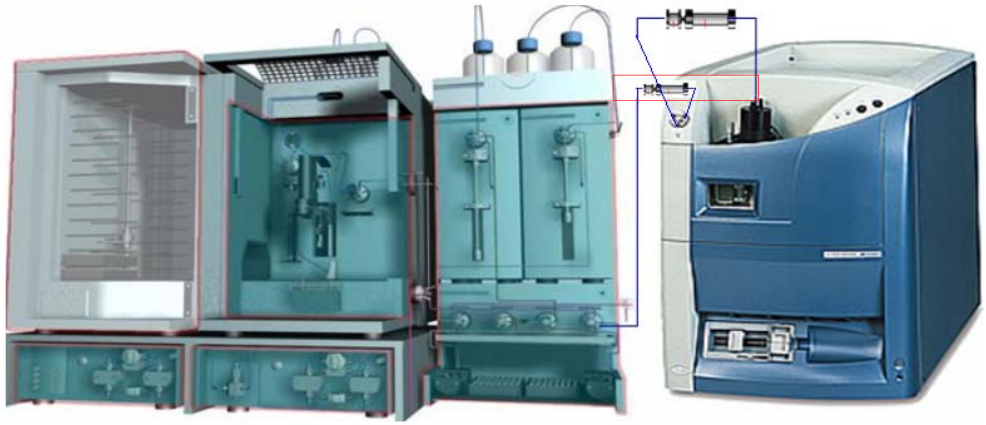
⋮

SPE-LC-MS/MS

Chromatographic conditions:

Elution step: High organic and strongly basic

SPE-LC-MS/MS



Chromatographic conditions:

Elution step: High organic and strongly basic

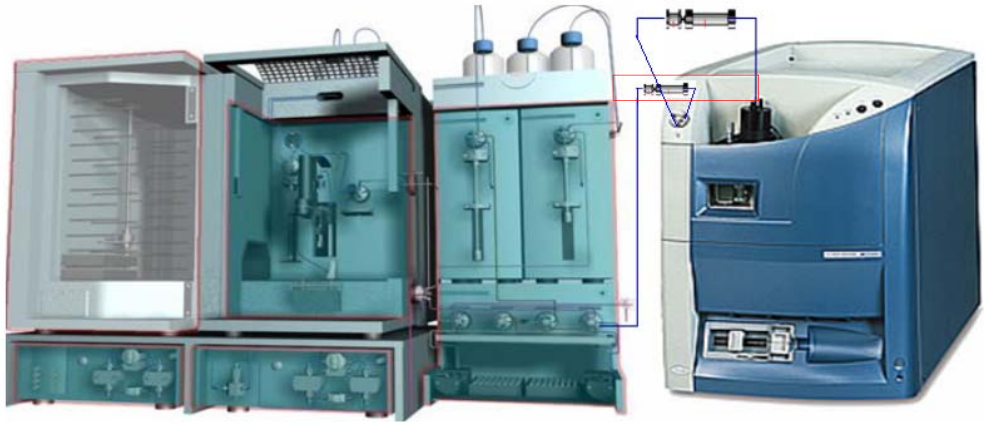
Column Gemini C_{18} : 5u 2x50mm

Mobile phase:

A: water 0.1% FA
B: Methanol 0.1% FA

Run: 10 min

SPE-LC-MS/MS



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Mobile phase:

A: water 0.1% FA
B: Methanol 0.1% FA

Run: **10** min

SPE + LC gradient!

SPE-LC-MS/MS

MRM transitions

Compound	Precursor ion (m/z)	Product ion (m/z)	Cone voltage	Collision energy (eV)
Ephedrine	166.0	147.9	20	10
		133.0		20
Amphetamine	135.9	118.9	20	8
		90.8		15
MDA	180.0	162.8	18	10
		134.9		18
PMA	166.0	149.0	20	12
		120.9		18
MDMA	194.0	162.8	20	10
		104.9		15
Methamphetamine	150.0	118.8	20	10
		90.8		15
MDEA	208.1	162.9	20	15
		104.9		25
Ephedrine-d_3	169.0	150.9	20	10
Amphetamine-d_{11}	146.9	97.9	20	15
MDA-d_5	185.0	167.9	18	10
MDMA-d_5	199.1	164.9	20	12
Methamphetamine-d_5	155.0	91.8	25	15
MDEA-d_6	214.1	165.9	25	12

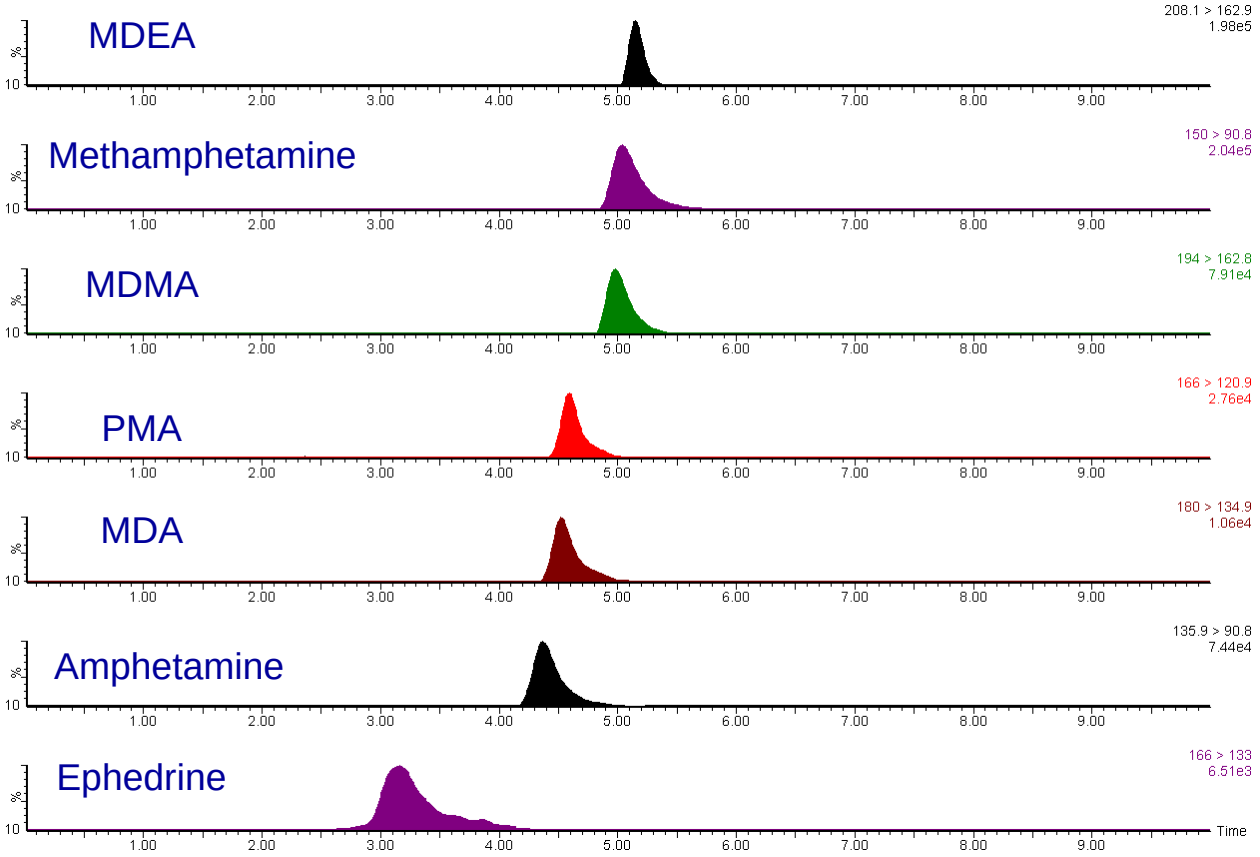
Quantifier: Most prominent precursor–product transition

Qualifier: The next most abundant

SPE-LC-MS/MS

Results

MRM chromatogram of the 7 amphetamines at 50 ng/ml in urine



SPE-LC-MS/MS

Results

Compound	LOD (ng/ml)	LOQ (ng/mL)	Equation	r^2
Ephedrine	5	50	y=0.127708x -0.64384	0.998693
Amphetamine	0.5	50	y=0.704004x -1.56333	0.997629
MDA	10	50	y=0.200421x -1.21882	0.999327
PMA	5	50	y=0.288939x +2.19461	0.995899
MDMA	5	50	y=0.405656x +2.98881	0.996181
Methamphetamine	5	50	y=1.37419x +3.28715	0.998409
MDEA	1	50	y=0.747869x -1.03843	0.998619

SPE-LC-MS/MS

Compound	Concentration (ng/mL)	Intra	Inter	Bias
Ephedrine	60	4.4	5.5	3.9
	800	5.7	6.9	-2.0
Amphetamine	60	8.2	7.3	-2.2
	800	6.0	5.3	2.1
	C1	10.5	9.6	13.4
	C3	4.6	4.1	15.6
MDA	60	5.8	7.5	-1.4
	800	9.1	7.3	-4.5
	C1	5.1	5.0	14.5
	C3	2.7	3.4	20.2
PMA	60	8.7	7.9	-0.7
	800	4.0	7.2	3.1
MDMA	60	11.3	9.0	-0.3
	800	9.9	8.6	-0.4
	C1	4.4	3.8	16.2
	C3	8.8	7.2	19.8
Methamphetamine	60	6.8	10.0	1.8
	800	8.8	7.5	3.2
	C1	0.9	1.1	18.3
	C3	3.4	8.7	16.0
MDEA	60	12.3	9.2	-0.4
	800	3.2	5.08	-0.9
	C1	1.9	2.1	17.2
	C3	3.1	4.4	11.9

- Intra- and inter-assay precision:

SPE-LC-MS/MS

- Stability:

  ✓ <u>in the autosampler (6 ± 2°C):</u>

  - blank samples were extracted and spiked at 800 ng/mL(n=6)
  - analysis after 24h (n=6)
  - before the analysis the IS were added
  - no statistical significant difference (P<0.01)

SPE-LC-MS/MS

- Matrix effect:

✓ First experiment

-Compare peak responses of urine samples spiked at 800 ngmL with mobile phase spiked at the same concentration

✓ Second experiment: post-column infussion

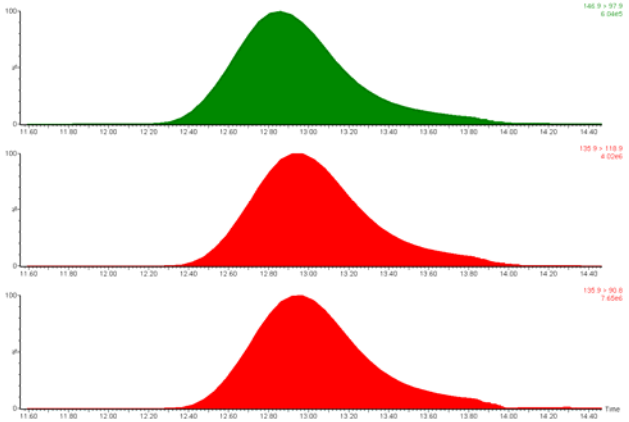
-Compare peak responses of urine samples spiked at 800 ngmL with mobile phase spiked at the same concentration

- no statistical significant difference ($P<0.01$)

SPE-LC-MS/MS

Analysis of authentic samples, previously screened using a routine FPIA method (TDX) for systematic toxicological analysis

Considered positifs when concentration higher than the established cut-off (200 ng/mL+ U)*



Most of them positifs for amphetamine

**Cut-off recommended by European/UK Laboratory Guidelines for Legally Defensible Workplace Drug Testing.*

Conclusions

- High-throughput of samples:
simultaneous extraction of the second sample in one clamp and the elution of the first sample in the second clamp optimal use of the extraction time

- No manual transfers: fully automated technique

- No need for a pre-concentration step: whole of the eluate is loaded onto the LC column

- Fully validated: linearity, precision, selectivity, specificity, matrix effect, stability

- Succesfully applied to authentic samples

Future

- Include other amphetamines and related compounds to this method: MDEA, mCPP, etc

- Application to other matrices: blood, hair, oral fluid

- Other methods: opiates, cocaine, benzodiazepines, THC

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