PHARMACEUTICALS and ILLICIT DRUGS by LC-MS

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OUTLINE

- Introduction:
  - Steps of a typical analytical procedure
  - Why LC-MS?
  - Interfaces
  - Analyzers
  - Identification points and confirmation criteria

- Examples of the application of:
  - HPLC-QqQ-MS/MS for the analysis of estrogens in water
  - HPLC-QqLIT-MS/MS for the:
    - Multiresidue analysis of 75 pharmaceuticals in water
    - Multiresidue analysis of 17 drugs of abuse in water
  - HPLC-QqTOF-MS/MS for the identification of degradation products of pharmaceuticals (ex. enalapril)

Conclusions
Chemical analysis of Phar. and IDs in water

- Complex matrices
- Low detection limits

Sensitive & selective analytical methods
- Long and laborious procedures

Sample pretreatm.
- filtration
- extraction
- centrifugation
- purification
- hydrolisis
- derivatization
- evaporation

Analysis
- LC (UV/FL/MS...)
- GC (FID/ECD/MS...)
- Bioassays

Chemical analysis of pharmaceuticals and illicit drugs

- Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is the technique of choice due to its sensitivity and selectivity.
  - GC is limited by analyte volatility and MW and, since most pharmaceuticals and illicit drugs, as well as their metabolites and transformation products, are polar, thermolabile and non volatile compounds it would require derivatization.
  - Bioassays require two analysis with an intermediate hydrolysis step for determination of conjugated and non-conjugated compounds.

- Triple quadrupole (QqQ) instruments are the most widely used although other MS/MS technologies, such as quadrupole-linear ion trap (QqLIT), quadrupole-time of flight (QqTOF) are gaining acceptance.

- These techniques accomplish the identification and confirmation criteria set by European Directives (96/23/EC), providing the number of IP required.
LC ion sources: APCI vs ESI

ESI most often used for pharmaceuticals and drugs

LC-MS/MS Analyzers

Quantitative target analysis
Identification/Confirmation
Structural Information

HPLC – Quattro LC (QqQ)™

ACQUITY UPLC™ – Q – TOF Micro™

HPLC – MDS Sciex (QTRAP)™

✓ SRM
✓ MS/MS scans
✓ Neutral loss scans
Waters Corporation (Manchester, UK)

✓ SRM
✓ MS scans
✓ TOF MS
✓ TOF MS/MS scans
Waters Corporation (Manchester, UK)

✓ SRM
✓ MS scans
✓ High sensitive MS/MS and MS scans
Applied Biosystems
Comparison of different LC-MS techniques

<table>
<thead>
<tr>
<th>Technique</th>
<th>Sensitivity</th>
<th>Selectivity</th>
<th>Mass accuracy</th>
<th>Dynamic range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q</td>
<td>Medium (SIM)</td>
<td>Low</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>QqQ</td>
<td>Medium (full scan) High (SRM)</td>
<td>High</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>TOF</td>
<td>Low</td>
<td>High</td>
<td>High</td>
<td>Low-Medium</td>
</tr>
<tr>
<td>IT</td>
<td>Medium (MS²)</td>
<td>High</td>
<td>Low</td>
<td>Low-Medium</td>
</tr>
</tbody>
</table>

LC-hybrid MS

<table>
<thead>
<tr>
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<th>Dynamic range</th>
</tr>
</thead>
<tbody>
<tr>
<td>QqLit</td>
<td>High (SRM)</td>
<td>High</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>QqTOF</td>
<td>Medium</td>
<td>High</td>
<td>High</td>
<td>Medium</td>
</tr>
</tbody>
</table>

Tandem mass spectrometry (MS/MS)

• Allows you to monitor the transition of precursor → product ion for each compound
• Transition is compound specific
• Eliminates background interferences (“noise”)
• Can obtain structural information of unknowns

Collision gas (N₂ or Ar)

QqQ

Quadrupole “Q1”

Collision cell “Q2”

Quadrupole “Q3”

Detector

Mixture of ions from column

Parent or precursor ion

Daughter or product ions
Identification and confirmation criteria for the analysis of drugs and other contaminants are defined in Directive 96/23/EC and Commission Decision 2002/657/EC, requiring a minimum of 3 identification points (4 for banned compounds = drugs of abuse).

<table>
<thead>
<tr>
<th>Technique</th>
<th>Number of IP earned per ion</th>
<th>Example per ions</th>
<th>IP earned</th>
</tr>
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<tbody>
<tr>
<td>LC-MS (Q)</td>
<td>1</td>
<td>SIM</td>
<td>1</td>
</tr>
<tr>
<td>LC-MS-MS (QqQ)</td>
<td>1 for precursor ion 1.5 for transition product</td>
<td>1 precursor 1 product (SRM)</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 precursor 2 products (2 SRM)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 precursors, each with 1 product (2 SRM)</td>
<td>5</td>
</tr>
<tr>
<td>LC-TOF-MS</td>
<td>2</td>
<td>1 ion</td>
<td>2</td>
</tr>
<tr>
<td>LC-QqTOF-MS</td>
<td>2 for precursor ion 2.5 for transition product</td>
<td>1 precursor 1 product (MS/MS)</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 precursor 2 products (MS/MS)</td>
<td>7.5</td>
</tr>
</tbody>
</table>

Specific criteria in LC-MS/MS: LC RT, 2 SRM transitions, ratio SRM transitions.

Examples of the application of LC-MS/MS to the analysis of pharmaceuticals in environmental samples:

- **HPLC-QqQ-MS/MS**
  - Analysis of estrogens in water

- **HPLC-QqLIT-MS/MS**
  - Multiresidue analysis of 75 pharmaceuticals in water
  - Multiresidue analysis of 17 drugs of abuse in water

- **UPLC-QqTOF-MS/MS**
  - Identification of degradation products of pharmaceuticals (ex. Enalapril)
Estrogens

- Estrogens are the most potent endocrine disrupting compounds known in terms of estrogenic activity.

- **Origin:**
  - natural (sex hormones)
  - synthetic

- **Uses:**
  - human medicine (contraceptives, management of menopausal and postmenopausal syndrome, prostatic & breast cancer, etc.)
  - veterinary (growth-promoters in livestock, 100% female populations in aquaculture)

- **Main sources** in the environment:
  - Excretion (urine as water-soluble conjugates, feces as “free” steroids)
  - STP (unefficient removal, run-off of sewage sludge used in agriculture)
**Estrogenic activity and effects**

Levels: pg-ng/L range

Biologically active concentrations:
as low as 0.1-1 ng/L (estradiol and ethynyl estradiol)several orders of magnitude higher than other analytes

Effects: - feminization- hermaphroditism- decreased fertility- ...

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**Target estrogens**

- **NATURAL HORMONES:**
  - Estradiol
  - Estriol
  - Estrone
  - Conjugates:
    - Estradiol-17-glucuronide
    - Estrone-3-sulfate
    - Estrone-3-glucuronide
    - Estriol-3-sulfate
    - Estriol-16-glucuronide

- **SYNTHETIC COMPOUNDS:**
  - Ethynyl estradiol
  - Diethylstilbestrol

*Selection based on:*
- Abundance in the human body
- Estrogenic potency
- Contraceptive
Analytical techniques for estrogen analysis

- **GC-MS** → needs derivatization
- **Biological assays** → high sensitivity
  - need hydrolysis of conjugated comp.
  - specific antisera
  - cross-reactivities
- **LC-MS** → direct analysis (no derivatization, hydrolysis)
  - on-line integration of sample prep. and enrichment
  - MS/MS ⇒ **Confidence in compound identification**
    ⇒ Improved sensitivity and selectivity
  - Matrix effects (ion suppression or enhancement)

Sequence of analytical methods developed for determination of estrogens in water

1/ Off-line SPE, LC-DAD-MS
2/ On-line SPE, LC-DAD
3/ On-line SPE, LC-MS/MS (QqQ)
**Analysis of estrogens in water - Evolution**

- Sample volume: 1 L → 20 mL
- SPE: off-line → SPE on-line
- LC column: long (25 cm) → short (12.5 cm)
- Detection: DAD/MS → MS/MS
- Selectivity
- Sensitivity: ng/L → pg/L
- Analysis time: 5-6 h → 45 min
- No automatization → Automatization
- No. target analytes: 5 → 10

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**Analysis of estrogens by on-line SPE-LC-MS/MS**

1. Conditioning (ACN+agua)
2. Sample preconcentration:
   - volume 20 mL
   - flow-rate 8 mL/min
3. Washing (HPLC water)
4. Elution

**Prospekt-2**

**On-line SPE**
- Cartridge (10 × 2 mm)
  - PLRP-s (15-25 µm)

**Analysis by LC-ESI-MS/MS**

- Column: Purospher STAR-RP-18e (125 x 2 mm, 5 µm)
- Mobile phase: gradient ACN/water
- Flow: 0.2 mL/min
- Detection: Electrospray (NI)
  - QqQ
  - SRM → 2 transitions per compound

On-line SPE optimization

Cartridge (10×2 mm):
- PLRP-s (15-25 µm)
- Hysphere Resin GP (10-12 µm)
- Hysphere Resin GP (8µm)
- Hysphere C₁₈ EC (8µm)

Loading flow rate:
- 5 mL/min
- 8 mL/min

pH adjustment of the sample:
- pH 5, 7, 9

Optimization of MS-MS exp. cond.

On column injection of st. mixtures:
- Full scan (m/z 50-500) → Identification of parent ion & selection of cone voltage
- Product-ion scan → Identification of the most abundant product ions and selection of optimum collision energies.

⇒ Product ion mass spectra of 271 (estradiol) and fragmentation pattern
Experimental conditions and validation

<table>
<thead>
<tr>
<th>Compuesto</th>
<th>Tiempo retención (min)</th>
<th>Transiciones SRM (m/z)</th>
<th>Cone (V)</th>
<th>Col. (eV)</th>
<th>LOD (ng/L)</th>
<th>% Rec.</th>
</tr>
</thead>
<tbody>
<tr>
<td>E3-3S</td>
<td>9.96</td>
<td>367 → 287</td>
<td>50</td>
<td>35</td>
<td>0.05</td>
<td>76.6</td>
</tr>
<tr>
<td>E3-16G</td>
<td>9.03</td>
<td>463 → 85</td>
<td>55</td>
<td>30</td>
<td>0.16</td>
<td>77.3</td>
</tr>
<tr>
<td>E2-17G</td>
<td>9.54</td>
<td>447 → 113</td>
<td>40</td>
<td>30</td>
<td>0.23</td>
<td>94.8</td>
</tr>
<tr>
<td>E1-3G</td>
<td>9.72</td>
<td>446 → 113</td>
<td>40</td>
<td>30</td>
<td>0.14</td>
<td>98.3</td>
</tr>
<tr>
<td>E1-3S</td>
<td>10.71</td>
<td>349 → 269</td>
<td>40</td>
<td>40</td>
<td>0.10</td>
<td>101.1</td>
</tr>
<tr>
<td>E1</td>
<td>11.54</td>
<td>287 → 171</td>
<td>50</td>
<td>40</td>
<td>0.54</td>
<td>94.9</td>
</tr>
<tr>
<td>E2</td>
<td>14.78</td>
<td>271 → 145</td>
<td>50</td>
<td>45</td>
<td>0.62</td>
<td>82.5</td>
</tr>
<tr>
<td>EE</td>
<td>15.72</td>
<td>295 → 145</td>
<td>50</td>
<td>40</td>
<td>2.21</td>
<td>81.5</td>
</tr>
<tr>
<td>E1</td>
<td>16.43</td>
<td>269 → 145</td>
<td>50</td>
<td>40</td>
<td>1.11</td>
<td>88.0</td>
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<tr>
<td>DES</td>
<td>17.05</td>
<td>267 → 222 (pico 1)</td>
<td>30</td>
<td>25</td>
<td>0.33</td>
<td>76.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>267 → 222 (pico 2)</td>
<td>30</td>
<td>25</td>
<td>1.00</td>
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Abreviations: E3-16G, estradiol-16-glucuronido; E3-3S, estradiol-3-sulfato; E1-3G, estrona-3-glucuronido; E1-3S, estrona-3-sulfato; E2-17G, estradiol-17-glucuronido; E3, estradiol; E1, estrona; E2, estradiol; EE, etinil estradiol; DES, diethylstilbestrol.

Analysis of a standard mixture of estrogens (25 ng/L) by on-line SPE-LC-MS/MS (QqQ). First window.
Analysis of a standard mixture of estrogens (25 ng/L) by on-line SPE-HPLC-MS/MS (QqQ). Second window.

Advantages of the method developed

- Sensitivity, selectivity, and reliability of results

- Minimum (filtration) or no sample preparation
- Full automation:
  - 15 samples + 6 cal. solns. + 1 blank
  - up to 192 analyses
- High throughput:
  - simultaneous SPE(n+1) and LC-MS-MS(n)
  - analysis time/sample = 45 min.

- Cost and time savings:
  - N2 for evaporation, eluting solvents
  - Low maintenance
  - Easy operation (no need for highly qualified staff)
  - Automatic data processing (Masslynx)

Minimum number of identification points required = 3 (4 for banned compounds)

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</table>

Example per ions

Technique

Identification and confirmation criteria

Analysis of estrone (0.68 ng/L) in river water (Llobregat)

Criteria for positive identification:
- retention time within 2%,
- SRM1/SRM2 ratio within ± 20%.

St. 0.5 ng/L

269>145 (SRM1 quant.)

269>143 (SRM2 confirm.)
Levels of estrogens along the treatment process in a ETAP

Not detected: estradiol, ethynyl estradiol, diethylstilbestrol, estriol, other conjug.

0.68
0.33
0.25
0.22

No risk
Estrogenic effects at conc. > 1-10 ng/ L

River Sand filtration Ozonation Activated carbon Drinking water

Examples of the application of LC-MS/MS to the analysis of pharmaceuticals in environmental samples

- HPLC-QqQ-MS/MS
  - Analysis of estrogens in water

- HPLC-QqLIT-MS/MS
  - Multiresidue analysis of 75 pharmaceuticals in water
  - Multiresidue analysis of 17 drugs of abuse in water

- UPLC-QqTOF-MS/MS
  - Identification of degradation products of pharmaceuticals (ex. Enalapril)
Target compounds - 75 pharmaceuticals

<table>
<thead>
<tr>
<th>Therapeutic groups</th>
<th>Compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Analgesics and anti-inflammatory agents</strong></td>
<td>Ketoprofen, Naproxen, Ibuprofen, Indomethacin, Diclofenac, Meclofenamic acid, Aspirin, Acetaminophen, ArSENiC (Acetaminophen) Phosphate, Phenytoin, Phenobarbital, Phenylbutazone, Codeine, Propranolol hydrochloride, Timolol, Betaxolol, Carazolol, Pindolol, Nadolol, Butalbital, Pentobarbital, Phenytoin, Carbamazepine</td>
</tr>
</tbody>
</table>
Analytical procedure

**SAMPLE PRE-TREATMENT**
- Filtration
  - 500 mL river water
  - 200 mL effluent
  - 180 mL influent

**EXTRACTION & PRE-CONCENTRATION**
- SPE

**INSTRUMENTAL ANALYSIS**
- HPLC-MS/MS (QqLIT)

**SPE OPTIMIZATION**
- Oasis HLB®
- Oasis MCX®
- Double Oasis HLB-MCX®

**Analytical Procedure**

- **Sample pH**
  - Natural sample pH
  - Sample pH=2-3
  - Sample pH=2-3

**SPE Optimization**

- **Macrolide Antibiotics**
  - % Recovery
- **Fluoroquinolone Antibiotics**
  - % Recovery
- **Other Compounds**
  - % Recovery

**Materials Used**
- Oasis HLB®
- Oasis MCX®
- Tandem Oasis HLB-MCX®
**LC-MS/MS (QqLIT)**

**Column:** Purospher ® RP-18 (125x2mm) (5 µm)
**Flow:** 0.2 mL/min NI i 0.3 mL/min PI

<table>
<thead>
<tr>
<th>Mobile phase</th>
<th>NI</th>
<th>PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent A: H₂O/ Solvent B: AcN / MeOH (1:1, v/v)</td>
<td>Solvent A: H₂O 0.1% HCOOH (pH= 2.5) / Solvent B: AcN</td>
<td></td>
</tr>
</tbody>
</table>

**Gradient**
- NI: 0 min (80%A-20%B), 0-20 min (20%A-80%B), 20-24 min (10%A-90%B), 24-27 min (10%A-90%B), 27-45 min (80%A-20%B)
- PI: 0 min (95%A-5%B), 0-25 min (5%A-95%B), 25-30 min (100%B), 30-35 min (95%A-5%B), 35-45 min (95%A-5%B)

**TOTAL RUN:** 40min

**400QTRAP™ Operational Parameters**
- **Interface Heater:** On
- **Resolution Q1:** Low
- **Resolution Q3:** Unit
- **Pause between mass ranges:** 2ms PI / 5ms NI
- **Dwell time:** 10 ms PI / 50 ms NI
- **Curtain Gas (CUR):** 30V
- **Collision Gas (CAD):** High
- **Ion Spray Voltage (IS):** 5500V PI / -4500V NI
- **Temperature (TEM):** 700°C
- **Ion Source Gas (GS1):** 50
- **Ion Source Gas (GS2):** 50

**PI Chromatogram**

**TIC of +MRM (124 pairs) 50 µg/L standard**

- Compounds monitored in one retention time window:
  - Phenazone type drugs and Codeine
  - Cholesterol lowering statin drugs and Fenofibrate
  - Psychiatric drugs
  - Ulcer healings and Histamine H₁ and H₂ receptor antagonists
  - Antibiotics
  - β-agonists and β-antagonists
  - Antihypertensives, diuretics, antidiabetics
  - Tamoxifen, metronidazole and clotrimazole
  - Internal standards: atenolol-d₇, carbamazepine-d₁₀, sulfamethoxazole-d₄, sulfathiazole-d₄, flumequine and ¹³C-Phenacetin.
- **Dwell time:** 10ms
- **Pause between mass ranges:** 2ms
- **Total Scan time:** 1.4880s
NI Chromatogram

Criteria for target analysis in MRM mode: LC RT, 2 MRM transitions, ratio MRM transitions

TIC of MRM (32 pairs) 50 µg/L standard

Compounds monitored in one retention time window

- Analgesics and anti-inflammatories except phenazone type
- Lipid regulators: Bezafibrate
- Barbiturates
- Furosemide and Chloramphenicol
- Internal standards: Ibuprofen-d₃, mecoprop-d₃ and Phenobarbital-d₅
- Dwell time: 50ms
- Pause between mass ranges: 5ms
- Total Scan time: 1.760s

QqLIT operation modes

Q3 as a quadrupole analyzer

Scan Type | Q1 | Q2 | Q3
---|---|---|---
Q3 Scan | Resolving Scan | RF-only | RF-only
Product Ion Scan (PS) | Resolving (Fixed) | Fragment | Resolving (Scan)
Precursor Ion Scan (PI) | Resolving (Scan) | Fragment | Resolving (Fixed)
Neutral Loss Scan (NL) | Resolving (Scan) | Fragment | Resolving (Scan Offset)
Selected Reaction Monitoring (SRM) | Resolving (Fixed) | Fragment | Resolving (Fixed)

Q3 as an ion trap analyzer

Scan Type | Q1 | Q2 | Q3
---|---|---|---
Enhanced Product Ion Scan (EPS) | Resolving (Fixed) | Fragment | Trap/Scan
MS³ | Resolving (Fixed) | Fragment | Isolation/frag trap/scan
Time delayed frag capture Product Ion (TDF) | Resolving (Fixed) | Trap/No frag | Trap/frag scan
Enhanced Q3 single MS (EPM) | RF-only | No frag | Trap/Scan
Enhanced Resolution Q3 Single MS (ERMS) | RF-only | No frag | Trap/Scan
Enhanced Multiply (EPI) | RF-only | No frag | Trap/empty scan
In an Information Dependent Acquisition (IDA) experiment, a MS survey scan is used to generate a peak list of all ions present, which is subjected to some criteria to filter out unwanted precursor ions. Then, the remaining ions are submitted for a MS/MS scan.

**IDA Criteria**

- Selected MRM transition above a given threshold of 5000 cps
- LIBRARY SEARCHING
- Survey Scan (MRM for 68 compounds in PI, 17 in NI and IS)

**DEPENDENT SCAN**

- EPI with three CE: +25, +35, +45 eV and -10, -20, -40 eV
- Dwell time of 15 ms for PI and 50 ms for NI
- Pause time of 2 ms
- Q1 Resolution Low
- Q3 Resolution Unit
- Scan rate: 4000 amu/sec
- Dynamic fill time: 20 ms

**Identification of the antibiotic Tylosin in an influent WWTP sample**

- TIC OF ALL MRM TRANSITIONS RECORDED
- XIC FOR TYLOSIN 916>174
- EPI (MS/MS) SCAN at CE= 25 eV
- EPI (MS/MS) SCAN at CE= 40 eV
- EPI (MS/MS) SCAN at CE= 55 eV
Tylosin spectra at CE=25 eV

Tylosin spectra at CE=55 eV

- The fit and RevFit value: information about similarity of the signals in the reference spectrum with those in the unknown samples.
- Purity Fit is a combination of both values.

Comparison with library
### Method validation - Recoveries

<table>
<thead>
<tr>
<th>Therapeutic groups</th>
<th>% Recovery surface water (n=3)</th>
<th>% Recovery WWTP effluent (n=3)</th>
<th>% Recovery WWTP influent (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analgesics and anti-inflammatories</td>
<td>60-106</td>
<td>50-122</td>
<td>50-138</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>21 (±6)</td>
<td>71 (±8)</td>
<td>71 (±8)</td>
</tr>
<tr>
<td>Acetylsalicylic acid</td>
<td>40 (±6)</td>
<td>51 (±1)</td>
<td>10 (±1)</td>
</tr>
<tr>
<td>Lipid regulators and cholesterol lowering statin drugs</td>
<td>60-71</td>
<td>50-105</td>
<td>40-130</td>
</tr>
<tr>
<td>Atorvastatin calcium</td>
<td>30 (±5)</td>
<td>96 (±6)</td>
<td>55 (±6)</td>
</tr>
<tr>
<td>Histamine H1 and H2 receptor antagonists</td>
<td>60-74</td>
<td>50-81</td>
<td>80</td>
</tr>
<tr>
<td>Psychiatric drugs</td>
<td>60-102</td>
<td>50-112</td>
<td>52-90</td>
</tr>
<tr>
<td>Barbiturics</td>
<td>91-105</td>
<td>60-70</td>
<td>50-54</td>
</tr>
<tr>
<td>Sulfonamide antibiotics</td>
<td>53-101</td>
<td>80-117</td>
<td>80-98</td>
</tr>
<tr>
<td>Macrolide antibiotics</td>
<td>50-112</td>
<td>50-92</td>
<td>50-81</td>
</tr>
<tr>
<td>Fluoroquinolone antibiotics</td>
<td>60-116</td>
<td>60-100</td>
<td>70-122</td>
</tr>
<tr>
<td>Tetracycline antibiotics</td>
<td>50-80</td>
<td>50-112</td>
<td>50-98</td>
</tr>
<tr>
<td>ß-blockers</td>
<td>70-80</td>
<td>60-90</td>
<td>50-106</td>
</tr>
<tr>
<td>Sulfamethoxazole</td>
<td>60-70</td>
<td>63-105</td>
<td>63-77</td>
</tr>
<tr>
<td>Sulfamethazine</td>
<td>30 (±10)</td>
<td>25 (±3)</td>
<td>47 (±1)</td>
</tr>
</tbody>
</table>

### Method validation – Limits of detection

<table>
<thead>
<tr>
<th>Therapeutic groups</th>
<th>Compounds</th>
<th>River water</th>
<th>Effluent</th>
<th>Influent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analgesics and anti-inflammatories</td>
<td>Ketoprofen</td>
<td>1</td>
<td>34</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>Naproxen</td>
<td>1</td>
<td>14</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Ibuprofen</td>
<td>1</td>
<td>16</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>Diclofenac</td>
<td>0.2</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Mefenamic acid</td>
<td>2</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Acetaminophen</td>
<td>0.2</td>
<td>9</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>Acetylsalicylic acid</td>
<td>1</td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Codeine</td>
<td>0.1</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Lipid regulators and cholesterol lowering statin drugs</td>
<td>Clofibrate</td>
<td>0.01</td>
<td>0.5</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>Bezafrilate</td>
<td>0.03</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>Sulfamethazine</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Sulfamethoxazole</td>
<td>2</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>Barbiturics</td>
<td>Butalbital</td>
<td>0.1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Pentoobarbital</td>
<td>8</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Phenobarbital</td>
<td>0.3</td>
<td>5</td>
<td>7</td>
</tr>
</tbody>
</table>

River water: 0.01 – 1 ng/L  
Effluent: 0.1 – 34 ng/L  
Influent: 0.2 – 41 ng/L
Monitoring of pharmaceuticals in the Ebro river basin

WWTP monitored: influent and effluent wastewaters
River water downstream the WWTP

Compounds detected in WWTP samples

- 52 detected compounds of 75 analyzed
  - Phenybutazone, Nifuroxazide, Danofloxacin, Oxytetracycline, Chlortetracycline, Lisinopril, Tamoxifen, Reserpine, Levothroid, Pravastatin, Pravastatin, Ketoprofen, Naproxen, Mefenamic acid, Butalbital, Pentobarbital, Phenobarbital, Ritonavir, and Lansoprazole were never detected.
**Elimination in WWTP**

- good elimination of NSAIDs
- generally poor elimination of other groups

**Compounds detected in river water**

- 43 detected compounds of 75 analyzed

<table>
<thead>
<tr>
<th>Compounds</th>
<th>% Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ofloxacin</td>
<td>100</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>80</td>
</tr>
<tr>
<td>Propyphenazone</td>
<td>60</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>40</td>
</tr>
<tr>
<td>Codeine</td>
<td>30</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>20</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>10</td>
</tr>
<tr>
<td>Bezafibrate</td>
<td>5</td>
</tr>
<tr>
<td>Hydrochlorothiazide</td>
<td>4</td>
</tr>
<tr>
<td>Sulfamethoxazole</td>
<td>3</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>2</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>1</td>
</tr>
<tr>
<td>Propranolol</td>
<td></td>
</tr>
<tr>
<td>Clofibric acid</td>
<td></td>
</tr>
<tr>
<td>Metronidazole</td>
<td></td>
</tr>
<tr>
<td>Glibenclamide</td>
<td></td>
</tr>
<tr>
<td>Diclofenac</td>
<td></td>
</tr>
<tr>
<td>Metoprolol</td>
<td></td>
</tr>
<tr>
<td>Atenolol</td>
<td></td>
</tr>
<tr>
<td>Gemfibrozil</td>
<td></td>
</tr>
<tr>
<td>Lorazepam</td>
<td></td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>100</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>80</td>
</tr>
<tr>
<td>Propyphenazone</td>
<td>60</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>40</td>
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<tr>
<td>Codeine</td>
<td>30</td>
</tr>
<tr>
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</tr>
<tr>
<td>Acetaminophen</td>
<td>10</td>
</tr>
<tr>
<td>Bezafibrate</td>
<td>5</td>
</tr>
<tr>
<td>Hydrochlorothiazide</td>
<td>4</td>
</tr>
<tr>
<td>Sulfamethoxazole</td>
<td>3</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>2</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>1</td>
</tr>
<tr>
<td>Propranolol</td>
<td></td>
</tr>
<tr>
<td>Clofibric acid</td>
<td></td>
</tr>
<tr>
<td>Metronidazole</td>
<td></td>
</tr>
<tr>
<td>Glibenclamide</td>
<td></td>
</tr>
<tr>
<td>Diclofenac</td>
<td></td>
</tr>
<tr>
<td>Metoprolol</td>
<td></td>
</tr>
<tr>
<td>Atenolol</td>
<td></td>
</tr>
<tr>
<td>Gemfibrozil</td>
<td></td>
</tr>
<tr>
<td>Lorazepam</td>
<td></td>
</tr>
</tbody>
</table>

*Danofloxacin, Enrofloxacin, Tetracycline, Doxycycline, Oxytetracycline, Chlorotetracycline, Lisinopril, Enalapril, Tamoxifen, Ciprofloxacin, Tetracycline, Oxacillin, Tylosin, Tilmicosin, Phenylbutazone, Nifuroxazide, Paroxetine, Famotidine, Betaxolol, Carazolol, Mevastatin, Pravastatin, Indomethacin, Mefenamic acid, Omeprazole, Lansoprazole and Clotrimazole were never detected.*
Range of concentrations found for the most representative individual compounds belonging to each therapeutic group.

Examples of the application of LC-MS/MS to the analysis of pharmaceuticals in environmental samples

- HPLC-QqQ-MS/MS
  - Analysis of estrogens in water

- HPLC-QqLIT-MS/MS
  - Multiresidue analysis of 75 pharmaceuticals in water
  - Multiresidue analysis of 17 drugs of abuse in water

- UPLC-QqTOF-MS/MS
  - Identification of degradation products of pharmaceuticals (ex. Enalapril)
Target drugs of abuse

### Amphetamine-like compounds

<table>
<thead>
<tr>
<th>Amphetamine-like compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphetamine</td>
</tr>
<tr>
<td>MDMA</td>
</tr>
<tr>
<td>Methamphetamine</td>
</tr>
<tr>
<td>R,R Pseudoephedrine</td>
</tr>
<tr>
<td>1S, 2R (+) Ephedrine D₃ hydrochloride</td>
</tr>
</tbody>
</table>

### Opiates

<table>
<thead>
<tr>
<th>Opiates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heroin</td>
</tr>
<tr>
<td>Morphine</td>
</tr>
<tr>
<td>6-Acetylmorphine</td>
</tr>
<tr>
<td>Morphine 3-ß-D-gluc.</td>
</tr>
<tr>
<td>Morphine 6-ß-D-gluc.</td>
</tr>
</tbody>
</table>

### LSD

<table>
<thead>
<tr>
<th>LSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSD</td>
</tr>
<tr>
<td>Nor-LSD &amp; nor-iso-LSD</td>
</tr>
<tr>
<td>2-oxo-3-hydroxy LSD</td>
</tr>
<tr>
<td>LSD - D₃</td>
</tr>
</tbody>
</table>

### Cocainics

<table>
<thead>
<tr>
<th>Cocainics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cocaine</td>
</tr>
<tr>
<td>Cocaethylene</td>
</tr>
<tr>
<td>Benzoylecgonine</td>
</tr>
</tbody>
</table>

### Cannabinoids

<table>
<thead>
<tr>
<th>Cannabinoids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ⁹-THC</td>
</tr>
<tr>
<td>11-hydroxy-THC</td>
</tr>
<tr>
<td>11-Nor-9-Carboxy-Δ⁹-THC</td>
</tr>
<tr>
<td>Δ²-THC – D₃</td>
</tr>
</tbody>
</table>

### Scheme analytical procedure

1. Conditioning solvents (ACN+water)
2. Sample preconcentration:
   - volume 5mL
   - flow 1 mL/min
3. Cartridge washing (HPLC water)
4. Elution (mobile phase)

**Prospekt-2** (Spark Holland)

**On-line SPE**

- Cartridge (10 × 2 mm):
  - PLRP-s for all analytes but cannabinoids (detected in PI)
  - Oasis HLB-s for cannabinoids (detected in NI)

**LC-ESI-(QqLIT)-MS/MS analysis**

- Column: Purospher STAR-RP-18e (125 x 2 mm, 5 µm)
- Mobile phase: linear gradient ACN/water
- Flow rate: 0.3 mL/min
- Injection: Electrospray (PI and NI)
- MRM ⇒ 2 transitions per compound (4 IPs)

**4000 Q-Trap** (Applied Biosystems)

**Detection**:

- Electrospray (PI and NI)
- MRM ⇒ 2 transitions per compound (4 IPs)
### Optimized SRM Conditions

<table>
<thead>
<tr>
<th>Target compound</th>
<th>Abbrev.</th>
<th>Retention (s)</th>
<th>LOD (ng/L)</th>
<th>LSD (ng/L)</th>
<th>AR (%)</th>
<th>RR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPH</td>
<td>EPH-d5(IS)</td>
<td>4.96 ± 0.06</td>
<td>286.2</td>
<td>311.3</td>
<td>80</td>
<td>141</td>
</tr>
<tr>
<td>AM</td>
<td>AM-d5(IS)</td>
<td>4.96 ± 0.06</td>
<td>286.2</td>
<td>311.3</td>
<td>80</td>
<td>141</td>
</tr>
<tr>
<td>MDMA</td>
<td>MDMA-d5(IS)</td>
<td>4.96 ± 0.06</td>
<td>286.2</td>
<td>311.3</td>
<td>80</td>
<td>141</td>
</tr>
<tr>
<td>LSD</td>
<td>LSD-d5(IS)</td>
<td>4.96 ± 0.06</td>
<td>286.2</td>
<td>311.3</td>
<td>80</td>
<td>141</td>
</tr>
<tr>
<td>MCR</td>
<td>MCR-d5(IS)</td>
<td>4.96 ± 0.06</td>
<td>286.2</td>
<td>311.3</td>
<td>80</td>
<td>141</td>
</tr>
<tr>
<td>SACM</td>
<td>SACM-d5(IS)</td>
<td>4.96 ± 0.06</td>
<td>286.2</td>
<td>311.3</td>
<td>80</td>
<td>141</td>
</tr>
<tr>
<td>HER</td>
<td>HER-d5(IS)</td>
<td>4.96 ± 0.06</td>
<td>286.2</td>
<td>311.3</td>
<td>80</td>
<td>141</td>
</tr>
<tr>
<td>nor-THC</td>
<td>nor-THC-d5(IS)</td>
<td>4.96 ± 0.06</td>
<td>286.2</td>
<td>311.3</td>
<td>80</td>
<td>141</td>
</tr>
<tr>
<td>THC</td>
<td>THC-d5(IS)</td>
<td>4.96 ± 0.06</td>
<td>286.2</td>
<td>311.3</td>
<td>80</td>
<td>141</td>
</tr>
</tbody>
</table>

### Method performance

**Target compounds**: Compounds analysed in Positive Ionization (PI) Mode

**MRM transitions (m/z)**

- /EHP-IS: 201 → 152
- /AM-IS: 201 → 152
- /MDMA-IS: 201 → 152
- /LSD-IS: 201 → 152
- /MCR-IS: 201 → 152
- /SACM-IS: 201 → 152
- /HER-IS: 201 → 152
- /nor-THC-IS: 201 → 152
- /THC-IS: 201 → 152

### Matrix effects - Corrected by SS
Matrix effects

- Retention Time within 2%,
- MRM1/MRM2 ratio within ± 20%.

High matrix effects in sewage water

Internal standard quantitation using surrogate standards

Standard solution
50 ng/L

1st MRM transition in standard solution
2nd MRM transition in standard solution

I.S. (20 ng/L) in HPLC-grade water

El Prat STP sample
Effluent 07/07
61.1 ng/L

1st MRM transition in wastewater
2nd MRM transition in wastewater

Reconstructed ion chromatograms obtained from the on-line SPE-LC-ESI-(QqLIT)MS-MS analysis of drugs of abuse

Spiked (50 ng/L) HPLC grade water

POSITIVE IONIZATION MODE

NEGATIVE IONIZATION MODE
Levels of drugs of abuse in waste & surface waters

<table>
<thead>
<tr>
<th>Dose</th>
<th>Cocainics</th>
<th>Opiates</th>
<th>ATS</th>
<th>Cannabinoids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>STP Influent</td>
<td>STP Effluent</td>
<td>Surface river water</td>
<td></td>
</tr>
<tr>
<td>100 mg</td>
<td>4.14900</td>
<td>1.149</td>
<td>0.160</td>
<td></td>
</tr>
<tr>
<td>5-10 mg</td>
<td>9.7500</td>
<td>1.1597</td>
<td>3.295</td>
<td></td>
</tr>
<tr>
<td>20-40 mg</td>
<td>7.1-820</td>
<td>3.2-874*</td>
<td>0.6-78</td>
<td></td>
</tr>
<tr>
<td>125-160 mg</td>
<td>2.5-18.1*</td>
<td>nd</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>100-150 mg</td>
<td>4.49.7*</td>
<td>4-36.2*</td>
<td>0.5*10.1</td>
<td></td>
</tr>
<tr>
<td>25-250 ug</td>
<td>4.9-91.3*</td>
<td>4.9-206*</td>
<td>1.0*18</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dose</th>
<th>Amphetamines</th>
<th>Methamphetamine</th>
<th>MDA</th>
<th>LSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>40-60 mg</td>
<td>3.5236</td>
<td>3.227</td>
<td>3.266</td>
<td>3-266</td>
</tr>
<tr>
<td>7-10 mg</td>
<td>1-190</td>
<td>1.1-90</td>
<td>0.9-200</td>
<td>0.4-20</td>
</tr>
<tr>
<td>5-10 mg</td>
<td>2.598</td>
<td>0.5-267</td>
<td>0.2-29</td>
<td>0.5-16</td>
</tr>
<tr>
<td>125-160 mg</td>
<td>2.5-18.1*</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>100-150 mg</td>
<td>4.49.7*</td>
<td>4-36.2*</td>
<td>0.5*10.1</td>
<td>1.0*18</td>
</tr>
<tr>
<td>25-250 ug</td>
<td>4.9-91.3*</td>
<td>4.9-206*</td>
<td>1.0*18</td>
<td>1.0*18</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dose</th>
<th>THC</th>
<th>11-nor-9-carboxy-THC</th>
<th>2-cyclo-3-hydroxy-THC</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mg</td>
<td>nd</td>
<td>4.3*-96.2</td>
<td>8.4*-46.3*</td>
</tr>
<tr>
<td>5-10 mg</td>
<td>nd</td>
<td>0.9-11.2</td>
<td>4.8*-15.0*</td>
</tr>
<tr>
<td>20-40 mg</td>
<td>nd</td>
<td>0.4-34.1</td>
<td>nd-10.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dose</th>
<th>11-nor-9-carboxy-THC</th>
<th>2-cyclo-3-hydroxy-THC</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mg</td>
<td>4.3*-96.2</td>
<td>8.4*-46.3*</td>
</tr>
<tr>
<td>5-10 mg</td>
<td>0.9-11.2</td>
<td>4.8*-15.0*</td>
</tr>
<tr>
<td>20-40 mg</td>
<td>0.4-34.1</td>
<td>nd-10.7</td>
</tr>
</tbody>
</table>

Population, dose, excretion rate
Removal efficiency
Dilution factor, half-life

Monitoring studies
Occurrence of drugs of abuse and metabolites in Barcelona – STP EL Prat

**Cocaines**

- Inf Eff: 2-Jul to 8-Jul
- Mon to Sun
- STP Depurbaix - Barcelona
- ng/L
- Cocaine

**Opiates**

- Inf Eff: 2-Jul to 8-Jul
- Mon to Sun
- STP Depurbaix - Barcelona
- ng/L
- Opiates

**Amphetamine-like compounds**

- Inf Eff: 2-Jul to 8-Jul
- Mon to Sun
- STP Depurbaix - Barcelona
- ng/L
- Amphetamine-like compounds

**Cannabinoids**

- Inf Eff: 2-Jul to 8-Jul
- Mon to Sun
- STP Depurbaix - Barcelona
- ng/L
- Cannabinoids

---

**Cocaine consumption (BCN)**

**Day-by-day pattern of cocaine consumption (estimate from influent sewage water)**

- Kg/day/1,300,000 inhabitants
- Doses/day/1,000 inhabitants

- Basis for calculation: 100 mg CO/dose; 45% CO excreted as BE;
- Q = 285,000 m³/day; population served by the plant = 1,300,000 hab.)

- ~ 4 Kg/day
- ~ 40,000 doses/day
Examples of the application of LC-MS/MS to the analysis of pharmaceuticals in environmental samples

- HPLC-QqQ-MS/MS
  - Analysis of estrogens in water
- HPLC-QqLIT-MS/MS
  - Multiresidue analysis of 75 pharmaceuticals in water
  - Multiresidue analysis of 17 drugs of abuse in water
- UPLC-QqTOF-MS/MS
  - Identification of degradation products of pharmaceuticals (ex. Enalapril)

UPLC-Q-TOF

**Quantitative target analysis**
- Identification/Confirmation
- Structural Information

**HPLC – Quattro LC (QqQ)™**
- MRM
- MS/MS scans
- Neutral loss scans

**ACQUITY UPLC™ – Q – TOF Micro™**
- TOF MS
- TOF MS/MS scans

Waters Corporation (Manchester, UK)

**HPLC – MDS SCIEX (QTRAP)™**
- MRM
- MS3
- High sensitive MS/MS and MS scans

SCARCE course. Valencia. February 7-8, 2011
Identification of transformation products by UPLC-QqTOF-MS/MS

Photodegradation of Enalapril (antihypertensive)

- HPLC water
- Artificial freshwater
- HPLC water + humic acids
- or Buffer

Enalapril

Suntest XLS (Atlas)

Sampling every day and storage at -20°C

Mass Spectrometric analysis

Enalapril and its photodegradates

- m/z 377: 3.28
- m/z 209: 5.98 (D207B)
- m/z 347: 4.40 (D346)

- D207A
- D207B
- D346

Identification by UPLC-QqTOF-MS/MS
Degradation profile of enalapril

![Degradation profile of enalapril graph]

QqToF-MS spectrum of enalapril

\([m/z \ 377, \ MW \ 376]\) and its degrade D346 \([m/z \ 347]\)

- Photodegradate (D346) is 30 Da lighter than parent compound
- Loss of 74 Da, like parent compound
- Formation of \(m/z \ 234\), like parent compound
**QqToF-MS: accurate mass measurements of D346**

<table>
<thead>
<tr>
<th>Measured mass</th>
<th>Calculated mass</th>
<th>Elemental composition</th>
<th>Error [ppm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>347.1985</td>
<td>347.1971</td>
<td>C_{21}H_{26}N_{2}O_{4}</td>
<td>+4.0</td>
</tr>
<tr>
<td>273.1594</td>
<td>273.1603</td>
<td>C_{14}H_{16}N_{2}O_{2}</td>
<td>-3.4</td>
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<td>234.1504</td>
<td>234.1494</td>
<td>C_{14}H_{20}NO_{2}</td>
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<tr>
<td>160.1120</td>
<td>160.1186</td>
<td>C_{11}H_{14}N_{1}</td>
<td>-3.9</td>
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<tr>
<td>134.0978</td>
<td>134.0970</td>
<td>C_{11}H_{13}N_{1}</td>
<td>+4.7</td>
</tr>
</tbody>
</table>

**Structure of D346**

**QqToF-MS spectrum of enalapril [m/z 377, MW 376] and its degradate D207 [m/z 208]**

- Photodegradate has same ion mass as fragment ion m/z 208 in MS² spectrum of enalapril
- Fragmentation pattern very similar to the one of fragment ion m/z 234 formed by enalapril
QqToF-MS: accurate mass measurements of D207

<table>
<thead>
<tr>
<th>Measured mass</th>
<th>Calculated mass</th>
<th>Elemental composition</th>
<th>Error [ppm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>208.1329</td>
<td>208.1333</td>
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<tr>
<td>191.1068</td>
<td>191.7072</td>
<td>C₂H₉N⁺</td>
<td>-2.0</td>
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<tr>
<td>180.4361</td>
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<td>C₈H₉NO₂⁺</td>
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<tr>
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<td>C₅H₁₀N⁺</td>
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</tbody>
</table>

Structure of D208

Conclusions

- LC-MS/MS in its various modalities (QqQ, QqLIT, and QqTOF) is a very powerful technique for the quantitation and identification of, respectively, known and unknown pharmaceuticals.
- Identified contaminants represent only a portion of those potentially present and their overall risk significance is largely ignored.