From accurate mass to identified water pollutants - non-target screening approaches using accurate mass, statistical analysis and compound libraries

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Environmental Analytical Chemistry
Outline

Introduction

→ Water pollutants

LC/MS screening approaches

→ Definitions
→ Harmonization of workflows
→ LC/MS methods and data interpretation

Application examples from the river Rhine and its tributaries

Conclusions and recommendations
Water pollutants

- Water is the prerequisite for all life
- Anthropogenic activities affect water quality

Monitoring and measures to reduce contaminants and their transformation products are needed!
Micropollutants from WWTP effluents

- PPCPs enter rivers via WWTP effluents (Benzotriazoles, PFCs, Diclofenac…)
  $\rightarrow$ polar POPs (PPOPs)
- Effects on aquatic ecosystems (fish: diclofenac, estrogens, neuroactive cpds.)
- WFD: improve ecological and chemical status
  - eco-friendly “chemicals
  - Advanced WWT processes
Screening approaches

Target knowns
- Standards
- Quantitation
- LowRes MS

Suspect screening known unknowns
- HiRes MS
- Generic methods
- Suspect list of compounds

Nontarget screening unknowns
- HiRes MS
- Generic methods
- Data reduction
- Library search
- In-silico methods

TQ-MS

HiRes MS (Q-TOF, FTICR, ...)

Screening
Nontarget screening

- HiRes MS
- Generic methods
- Data reduction
- Library search
- *In-silico* methods

HiRes MS (Q-TOF, FTICR, ...)

Science 291, 5007 (2001) 1221-1224
Non-target screening

Separate and Detect

Feature Finding

Data Analysis

Compound Identification

Separate and detect

Find peaks and quantitate

Comparative or multivariate analysis

MH Qual or ProFinder

Identify peaks

MP or MPP

ID Browser (Q-TOF only)
Measurement

Devices:

**Liquid chromatography**
1260 Infinity System
(Agilent Technologies)

**High-resolution mass spectrometry**
6550 iFunnel Q-TOF
(Agilent Technologies)

**Measurement mode:** Full-scan

- **Electrospray-ionization** (ESI)
- **All ions** within a defined range
  (e.g. 100-1000 Da) are measured
Generic methods

Norman Association – NTS trial

→ Schymanski et al. (28 authors):
Non-target screening with high resolution mass spectrometry: Critical review using a collaborative trial on water analysis.

Waterchemical Society (GDCh) – Expert group on Non-target Screening

→ Large volume injection (100 µL)
# LC conditions

<table>
<thead>
<tr>
<th>Column</th>
<th>Dim. (mm); Particle size (µm)</th>
<th>Solvents</th>
<th>Flowrate (ml/min); Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agilent Poreshell HPH C(_{18})</td>
<td>2.1 x 150; 2.7</td>
<td>H(_2)O/MeOH (FA pos only)</td>
<td>0.3; 35 min</td>
</tr>
<tr>
<td>Agilent Zorbax Eclipse Plus C(_{18})</td>
<td>2.1 x 150; 3.5</td>
<td>H(_2)O/ACN (FA)</td>
<td>0.3; 37 min</td>
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<tr>
<td>Agilent Zorbax Extend C(_{18})</td>
<td>2.1 x 50; 1.8</td>
<td>H(_2)O/ACN (FA)</td>
<td>0.5; 46 min</td>
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<tr>
<td>Phenomenex Kinetex C(_{18})</td>
<td>3.0 x 100; 2.6</td>
<td>H(_2)O/MeOH (FA)</td>
<td>0.2; 41 min</td>
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<tr>
<td>Thermo Hypersil Gold</td>
<td>2.1 x 100; 3.0</td>
<td>H(_2)O/MeOH (FA)</td>
<td>0.2; 35 min</td>
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<tr>
<td>Waters Acquity UPLC BEH C(_{18})</td>
<td>2.1 x 100; 1.7</td>
<td>H(_2)O/MeOH (FA)</td>
<td>0.3; 18 min</td>
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<tr>
<td>Waters Atlantis HSS T3</td>
<td>2.1 x 150; 3.5</td>
<td>H(_2)O/MeOH (FA)</td>
<td>0.2; 19 min</td>
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<tr>
<td>Waters C(_{18}) BEH</td>
<td>2.1 x 100; 1.7</td>
<td>H(_2)O/MeOH (NH(_4)FA, pH 5)</td>
<td>0.45; 13 min</td>
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### MS conditions

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Set points</th>
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</thead>
<tbody>
<tr>
<td>Scan range</td>
<td>100 to 1200 / 1700</td>
</tr>
<tr>
<td>Mass resolving power</td>
<td>20,000 to 50,000 / 100,000</td>
</tr>
<tr>
<td>Ionization</td>
<td>Electrospray</td>
</tr>
<tr>
<td>Fragmentation</td>
<td>CID, HCD</td>
</tr>
</tbody>
</table>

→ How much resolving power do we need?
→ How much mass accuracy?
Nontarget approach

Screening without Preliminary information

LC-HRMS

↓

Molecular formulae

↓

Chemical structures

Two independent physical properties of ions:
- Exact mass of a monoisotopic ion
- Relative isotopic abundances (RIA; M+1, M+2)

Marshall A., PENAS 2008
**Accuracy**: The proximity of the experimental measurement to the true value (exact mass).

**Precision**: The repeatability of measurements reflecting random errors.

Brenton & Godfrey, JASMS 2010
Accurate mass measurement

\[ \sigma_{ppm} = \frac{10^6}{C \cdot R \cdot \sqrt{S}} \]

Factors for mass accuracy
- Tuning of mass axis
- Fine tuning
- Lock mass correction
- Ion statistics
- Chemical background

Brenton & Godfrey, JASMS 2010; Blom, Anal. Chem. 2001
Which accuracy needed?

Number of possible compositions
- increases exponentially with increasing mass
- increases linearly with mass error
- is a function of mass defect

**CHNO:** unique formula at
- m/z 118 → 34 ppm (4 mDa)
- m/z 750 → 0.018 ppm (0.013 mDa)

Grange et al. RCM 2002
Mass resolving power

Resolving mass doublets in complex mixtures

- N2/C2H4 \( \Delta m = 25 \text{ mDa} \) \( \Rightarrow \) \( R = 20 \, 000 \) \( (m/z \, 500) \)
- S/O2 \( \Delta m = 17.7 \text{ mDa} \) \( \Rightarrow \) \( R = 28 \, 000 \) \( (m/z \, 500) \)
- C3/SH4 \( \Delta m = 3.4 \text{ mDa} \) \( \Rightarrow \) \( R = 147 \, 000 \) \( (m/z \, 500) \)

\( \Rightarrow \) isotope pattern

9.4-T FT-ICR-MS
\( R = 400 \, 000 \)

European Crude Oil
\( (+) \) ESI FT-ICR MS
## MS/MS mass accuracy (6550 QTOF)

<table>
<thead>
<tr>
<th>Hydrochlorothiazide</th>
<th>precursor</th>
<th>product ion 1</th>
<th>product ion 2</th>
<th>product ion 3</th>
<th>product ion 4</th>
<th>product ion 5</th>
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<tbody>
<tr>
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<td>295.9572</td>
<td>268.9463</td>
<td>231.9953</td>
<td>204.9844</td>
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<td>231.9950</td>
<td>204.9841</td>
<td>126.0114</td>
<td>77.9655</td>
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<td>9902</td>
<td>384</td>
<td>9547</td>
<td>3134</td>
<td>21293</td>
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<tr>
<td></td>
<td>19.1</td>
<td>46.5</td>
<td>1.8</td>
<td>44.8</td>
<td>14.7</td>
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<tr>
<td></td>
<td>-1.7 ppm</td>
<td>-0.3 ppm</td>
<td>-1.4 ppm</td>
<td>-1.6 ppm</td>
<td>-1.6 ppm</td>
<td>-0.3 ppm</td>
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<table>
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<tr>
<th>PFNA</th>
<th>precursor</th>
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<th>product ion 2</th>
<th>product ion 3</th>
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<tr>
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<td>462.9632</td>
<td>418.9734</td>
<td>218.9862</td>
<td>168.9894</td>
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<td>N.A.</td>
<td>418.9722</td>
<td>218.9860</td>
<td>168.9894</td>
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</tr>
<tr>
<td>not detected</td>
<td>2866</td>
<td>9890</td>
<td>14545</td>
<td></td>
</tr>
<tr>
<td></td>
<td>19.7</td>
<td>68.0</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-2.9 ppm</td>
<td>-0.8 ppm</td>
<td>0.2 ppm</td>
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</table>

<table>
<thead>
<tr>
<th>Clarithromycin</th>
<th>precursor</th>
<th>product ion 1</th>
<th>product ion 2</th>
<th>product ion 3</th>
<th>product ion 4</th>
<th>product ion 5</th>
<th>product ion 6</th>
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<tbody>
<tr>
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<td>748.4842</td>
<td>716.4580</td>
<td>590.3898</td>
<td>558.3637</td>
<td>158.1176</td>
<td>116.1070</td>
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<td></td>
<td>1376</td>
<td>0</td>
<td>3537</td>
<td>998</td>
<td>11982</td>
<td>1845</td>
<td>1962</td>
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<tr>
<td>11.5</td>
<td>29.5</td>
<td>8.3</td>
<td>100.0</td>
<td>15.4</td>
<td>16.4</td>
<td></td>
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<tr>
<td>0.4 ppm</td>
<td>-0.8 ppm</td>
<td>1.8 ppm</td>
<td>-2.8 ppm</td>
<td>0.9 ppm</td>
<td>1.7 ppm</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Sampling sites

Small river in Southwest Germany
• Anthropogenically influenced mass fluxes
### Measurement

#### SPE
250 mL $\rightarrow$ 0.5 mL, ENV+ (ASPEC, Gilson)

#### HPLC
Agilent 1290 Infinity UHPLC System  
Zorbax Eclipse Plus C-18 column (160 x 2.1 mm, 1.8 µm)  
Gradient: linear, water (0.1% formic acid) - methanol

#### MS
Agilent 6550A iFunnel Q-TOF-MS  
2 GHz extended-dynamic mode  
(20,000 @ m/z 650; < 1 ppm)  
Mass range 100 - 1600 Da

#### Software
Agilent Mass Hunter Qual B.06  
Forensics and toxicology library (> 7000 entries; Agilent)  
Pesticide database (1600 entries; Agilent)
Suspect screening

Known unknowns

- HiRes MS
- Generic methods
- **Suspect list of compounds**
  50 water pollutants

Basepeak chromatograms

downstream of WWTP

upstream of WWTP

<table>
<thead>
<tr>
<th>Compound list created in a worksheet</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sum form.</strong></td>
</tr>
<tr>
<td>---------------</td>
</tr>
<tr>
<td>C10H11N3O3S</td>
</tr>
<tr>
<td>C16H25NO2</td>
</tr>
<tr>
<td>C9H7Cl2N5</td>
</tr>
<tr>
<td>C15H25NO3</td>
</tr>
<tr>
<td>C11H12N2O</td>
</tr>
</tbody>
</table>

or import in database manager
Find by Formula Search (suspect list)

Formula Source: 50 WP data base (10 ppm match) → 35 hits based on accurate mass and isotope pattern

Bisoprolol: score 83, Δmass – 1.34 ppm

Extracted ion chromatograms

Isotope pattern for C18H31NO4
Bisoprolol
Find by Formula Search (suspect list)

**Tris-(-2-chloroethyl) phosphate (TCEP):** score 99, \( \Delta \text{mass} -1.98 \text{ ppm} \)

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**Isotope pattern for C6H12Cl3O4P**

**Tris-(-2-chloroethyl) phosphate**
Considerable increase of Bisoprolol and TCEP due to input into the river from WTP effluents.
Identification and confirmation

Non-target HR-MS/(MS) Acquisition

Target list

Target Screening

Peak picking or XICs

Suspect list

Suspect Screening

Peak picking

Non-target Screening

Level 1  Confirmed Structure by reference standard

Level 2  Probable Structure by library/diagnostic evidence

Level 3  Tentative Candidate(s) suspect, substructure, class

Level 4  Unequivocal Molecular Formula insufficient structural evidence

Level 5  Mass of Interest multiple detection, trends, ...

Increasing identification confidence
“downgrading” with contradictory evidence

Compound confirmation

- Pure standards (mass, retention time)
- Mass fragmentation (accurate mass)
  - Targeted MS/MS run for a list of compounds
    (retention time, precursor mass, CE)
  - Comparison with library spectra (Agilent ForensicsTox library)
Nontarget screening

Accurate mass measurement

Automated peak detection (MFE)

Statistical data analysis

Molecular formula generation

Identification

MS-MS fragmentation

MS/MS database or library

Chemical database and in-silico fragmentation
Data evaluation - deconvolution

Scan 15.04 – 15.15 min

EIC m/z 237.1016

Molecular feature spectrum

Cpd 37: 15.08 236.0944: +ESI MFE Spectrum (14.99-15.21 min) Frag=400.0V A8-7-r003.d

Counts (%) vs. Mass-to-Charge (m/z)

Counts vs. Acquisition Time (min)
Data evaluation - deconvolution

Peak deconvolution or picking

- Thresholds
- Ranges
- Chromatographic parameters

Peaks in EI chromatograms

EI chromatograms of 25 compounds
Peak finding (MFE)

<table>
<thead>
<tr>
<th>Mass</th>
<th>RT (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>113.121</td>
<td>5.97</td>
</tr>
<tr>
<td>114.047</td>
<td>4.52</td>
</tr>
<tr>
<td>114.066</td>
<td>5.43</td>
</tr>
<tr>
<td>114.116</td>
<td>0.79</td>
</tr>
<tr>
<td>114.116</td>
<td>0.78</td>
</tr>
<tr>
<td>115.063</td>
<td>0.51</td>
</tr>
<tr>
<td>115.063</td>
<td>0.64</td>
</tr>
<tr>
<td>116.031</td>
<td>0.81</td>
</tr>
<tr>
<td>116.985</td>
<td>0.56</td>
</tr>
<tr>
<td>117.042</td>
<td>5.6</td>
</tr>
<tr>
<td>117.079</td>
<td>0.19</td>
</tr>
</tbody>
</table>

Export data
→ Compound exchange format (cef)

Data evaluation
Mass Profiler or
Mass Profiler Professional
→ Peak alignment
→ Number of ions > 2
→ Filtering
  frequency, n-fold change
  Blank
Internal standards

Diclofenac-d₄ in ESI Pos (m/z 300.04907)

100 injections

Diclofenac_d4

Zoom in
Rhine sampling

- Rhine sampling campaign 08/2014
- A. Fath, FH Schwenningen
- Grab samples
- Filtration → 100µL injection
- LC-ESI-QTOF measurement (3 replicates)
- MFE → export to Mass Profiler Professional
MPP – Data filtering

3738 compounds aligned (pos)

Filter by flag:

→ only compounds in at least 2 replicates → 3003
→ Abundance > 5000 counts → 2694
→ In at least 2 samples → 2122
→ **Up-regulated in Rhine vs. Blank 1453**
→ Unique to Rhine 1308
Boxplot of selected features
PCA plot
Lamotrigine in Rhine samples
Similar behavior

Similar entities 255.0077 @ 6.8 min (Lamotrigine, anticonvulsant)
Euclidean distance $0.9 \leq r \leq 1.0$

→ 10 compounds

Lake Constance

Lahne confl.

Wesel confl.

$\log_2$ (Normalized Abundance)

→ Sample No. 1 … 25
Similar behavior

Features at: 171.1259 @ 4.1min; 258.077 @ 9.6 min
Identification

→ Export for identification
→ Library search (Agilent Forensics Tox)
→ Accurate mass and isotope pattern → Fragmentation match

Gabapentin, anticonvulsant, analgesic
Identification

Carbamazepine

Cpd 9: Carbamazepine; C15 H12 N2 O; 9.564; + FBF Spectrum (rt: 9.564 min)

mass spectrum

m/z 259.0845 ([C15 H12 N2 O]+Na)+

m/z 260.0880 ([C15 H12 N2 O]+Na)+

m/z 261.0780 ([C15 H12 N2 O]+Na)+

m/z 259.259.4 259.6 259.8 260 260.2 260.4 260.6 260.8 261 261.2

Counts vs. Mass-to-Charge (m/z)
QTOF screening approaches enable to

- characterize surface water quality
- detect relevant pollutants
- retrieve relevant metabolites (all ions)
- requires
  - statistical approaches to reduce data
  - library data AMRT for ID