New Algorithms using All Ions MS/MS

Identification of isomeric drugs/drugs metabolites in Forensic samples

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Director LCMS Applications
Applied Markets
Agilent Technologies, Santa Clara, US
Agenda

• Agilent forensic solutions
• Application Kits for forensic
• High resolution mass spec for identification and quantitation
• All Ions MS/MS
  • Easy setup
  • Identification with data-independent MS/MS acquisition
• Summary
Forensic Solutions
For Legally Defensible Results

Forensic customers need high quality results quickly:
• Must withstand legal scrutiny
• Need for robustness, efficiency
• Need confidence in results
• Increased use of Accurate Mass

What Agilent offers:
• Complete methods with proven performance
• Application kits for fast startup to maximize efficiency
• Superior software for routine testing
The toolbox for accurate mass screening
The complete solution from Agilent
Accurate Mass LC/MS Application Kits
Untargeted Screening with TOF or Q-TOF

- **Pesticides**
  - Test Mix: 253 compounds
  - DB: 1600+ compounds
  - Library: 700+ compounds

- **Veterinary Drugs**
  - Test Mix: 146 compounds
  - DB: 1000+ compounds
  - Library: 600+ compounds

- **Forensic Toxicology**
  - Test Mix: 139 compounds
  - DB: 9000+ compounds
  - Library: 3000+ compounds
Screening workflow using LC/Q-TOF MS

Instrumentation

Technology Innovation

- Dual-stage ion mirror (resolution)
- Longer flight tube (resolution)
- INVAR flight tube (mass accuracy)
- ADC (dynamic range)
- 4 GHz electronics (resolution, mass accuracy, sensitivity, dynamic range)
- Ion Beam Compression Technology (resolution + mass accuracy)
- Orthogonal spray source (signal-to-noise)
- iFunnel technology (sensitivity)
- Ion acceleration in hexapole collision cell (faster MS/MS spectra)

Webinar Separation Science - Vet Drugs
July 25, 2013
Why do you need a QTOF?

- Non-targeted screening
- Retrospective data analysis
- Unknown metabolite identification
- Spectral information add highest analytical sensitivity levels
- Elimination of errors
Agilent Unique Concept

**Issues**
- MS only on TOF is powerful but reveals too many errors
- Matrix might influence mass accuracy and therefore the quality of the identification

**Solutions**
- Retention time can help to distinguish between isomers
- Isotopic calculation as additional parameter for the MS identification
- The Broecker, Herre & Pragst high mass accurate MS/MS spectra library for forensic toxicology

**Innovative Workflow**
- Acquire data in data-dependent MS/MS mode
- Processes to analyse accurate MS and accurate MS/MS data
- Combine results from MS database search, molecular formula generation and MS/MS library searching
Structural diversity, example: M = 149

Number of isomers according to MOLGEN
(Molecular structure generation; http://molgen.de)

<table>
<thead>
<tr>
<th>Formula</th>
<th>No. Isomers (MOLGEN)</th>
<th>Number Beilstein</th>
<th>Number NIST</th>
<th>Exist</th>
<th>Accurate mass</th>
<th>M</th>
<th>M+1</th>
<th>M+2</th>
<th>M+3</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₃H₆NO₆</td>
<td>41580</td>
<td>0</td>
<td>0</td>
<td>NO</td>
<td>148,996038</td>
<td>100</td>
<td>3,89</td>
<td>1,29</td>
<td>0,05</td>
</tr>
<tr>
<td>C₂H₃N₃O₅</td>
<td>152977</td>
<td>0</td>
<td>0</td>
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<td>149,007271</td>
<td>100</td>
<td>3,51</td>
<td>1,08</td>
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<tr>
<td>C₃H₆NO₃</td>
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<td>3</td>
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<td>149,011293</td>
<td>100</td>
<td>8,1</td>
<td>0,9</td>
<td>0,06</td>
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<tr>
<td>CH₃N₅O₄</td>
<td>99306</td>
<td>0</td>
<td>0</td>
<td>NO</td>
<td>149,018504</td>
<td>100</td>
<td>3,13</td>
<td>0,86</td>
<td>0,03</td>
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<tr>
<td>C₆H₃N₂O₂</td>
<td>50459744</td>
<td>2</td>
<td>0</td>
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<td>149,022526</td>
<td>100</td>
<td>12,31</td>
<td>0,69</td>
<td>0,02</td>
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<td>C₁₁H₃N</td>
<td>53109027</td>
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<td>4,99</td>
<td>1,13</td>
<td>0,05</td>
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<td>7,34</td>
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<tr>
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<tr>
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<tr>
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<td>0</td>
<td>NO</td>
<td>149,066120</td>
<td>100</td>
<td>6,09</td>
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<tr>
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<td>100</td>
<td>10,31</td>
<td>0,68</td>
<td>0,03</td>
</tr>
</tbody>
</table>

C₉H₁₁NO 25 895 621 724 45 YES 149,084060 100 10,31 0,68 0,03

<table>
<thead>
<tr>
<th>Included atoms:</th>
<th>Molecular formulas</th>
</tr>
</thead>
<tbody>
<tr>
<td>C, H, N, O, S, P, F, Cl, Br:</td>
<td>120</td>
</tr>
<tr>
<td>C, H, N, O, S, P:</td>
<td>38</td>
</tr>
<tr>
<td>C, H, N, O:</td>
<td>27</td>
</tr>
</tbody>
</table>

2 ppm = ± 0,0003
Examples of structural isomers of cathinone

(www.chemspider.com, 829 hits)

Altogether 25,895,621 structural isomers possible
Stereoisomers not included

C₉H₁₁NO
Agilent Unique Concept

Issues

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Solutions

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• The Broecker, Herre & Pragst high mass accurate MS/MS spectra library for forensic toxicology

Innovative Workflow

• Acquire data in data-dependent MS/MS mode
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• Combine results from MS database search, molecular formula generation and MS/MS library searching
Identification of two designer Drugs, Identification of two isomeric compounds MDMA and Methedron

Samples have been analyzed with LC-Method 1 in MS-Mode. Injection volumes between 5 µL. Injection solvent: Water/Acetonitril 95/5 + 0.1 % formic acid. Column type: 2.1 × 50 mm, 1.8 µm Eclipse Plus C18.

Sample prep urine sample

50 µL urine shipped as sample
Diluted (1:5) by adding 200 µL water
2nd dilution 10:1 using injection solvent

Samples:

U-DDLO
Relatively low concentration

U-DDHi
Relatively high concentration

Compounds:

![Structure of MDMA](image)

Name = MDMA
Molecular Formula = C₁₁H₁₅NO₂
LogP (ACD) = 1.81
CAS-No = 69610-10-2
Monoisotopic Mass = 193.110279 Da
[M+H]+ = 194.117555 Da
[M-H]- = 192.103002 Da

![Structure of Methedron](image)

Name = Methedron
Molecular Formula = C₁₁H₁₅NO₂
LogP (ACD) = 1.47
CAS-No = 530-54-1
Monoisotopic Mass = 193.110279 Da
[M+H]+ = 194.117555 Da

For Forensic Use.
Identification of two designer drugs results

Sample: U-DDhi
Inj-Vol: 2 µL

Full spectrum for both compounds

Isobaric compounds could be separated. Additional information such as logP values might help. Compound with lower value elutes earlier.
Identification of two designer drugs results

Sample: U-DDhi
Inj-Vol: 2 µL

Retention time match is part of calculated score and helps identifying isobaric compounds in MS1
Agilent Unique Concept

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Database with 9,000+ Compounds

- Designer Drugs
- Hallucinogens
- Stimulants
- Opioids
- Benzodiazepines
- Hypnotics
- Neuroleptics
- Barbituates
- Antidepressants
- Antiepileptics

- Accurate Mass Correction
- Molecular Structure
- Quality Controlled
- CAS Number/PubChem Link

Library spectra for 3,000+ Compounds
Database & Library of toxic forensic compounds

“Broecker, Herre & Pragst PCDL (Personal Compound Database & Library)”

contains all kinds of poisons:

Drugs of abuse
Medical drugs
Pesticides

Alkaloides
Toxic reagents
Other poisons

Deuterated standards

- Database, over 9000 substances: Text-based information of the compounds such as name, CAS-number, molecular and structural formula, neutral mono-isotopic mass, isotope pattern, retention time (optional).

- CID MS/MS spectral library, over 3000 substances: 3 spectra per compound at different collision energies

MS/MS spectra generated at CID energies of 10, 20 and 40 V

- Cleaned by background subtraction
- Controlled for structural plausibility
- Reviewed
Database & Library of toxic forensic compounds

“Broecker, Herre & Pragst PCDL (Personal Compound Database & Library)”

contains all kinds of poisons:

- Drugs of abuse
- Medical drugs
- Pesticides
- Alkaloides
- Toxic reagents
- Other poisons
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- **CID MS/MS spectral library, over 3000 substances:** 3 spectra per compound at different collision energies

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For Forensic Use.
**Agilent Unique Concept**

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**Innovative Workflow**
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- Combine results from MS database search, molecular formula generation and MS/MS library searching
Innovative Workflow – Combined ID algorithms

Find by Formula

- Compound information from formula or Database
- Spectral performance calculated

Ion Information
- Ion chromatogram drawn from information
- Spectra extracted from integrated peak

Formal Source | Formula Matching | Positive Ions | Negative Ions | Scoring | Results
---|---|---|---|---|---
- Source of formulas to confirm
  - Those formulas:
    - C16H22N2
  - Compound exchange file (CEF): ...
  - Database:
    - D:\MassHunterLibrary\FMVR Case.cdb ...
- Worklist
  - Database values to match
    - Mass
    - Mass and retention time (retention time optional)
    - Mass and retention time (retention time required)

July 25th, 2013
Forensic Seminar: All Ions MS/MS

For Forensic Use.
Innovative Workflow – Combined ID algorithms
More confidence via multiple ID algorithms

- New and unique combined “Find by FbF with MS/MS spectral extraction” from Auto MS/MS for automatic MS/MS library search with multiple CEs
- Unique combined scoring and view of results from AMRT DB search, MS/MS library search and MFG
- Overlays isotopic pattern for manual MFG, Find by Formula, AM DB search
- Reduction of errors
Example

• Combining ms and msms data mining
• Using the msms library to identify compounds
• Combine and weight results
• Example
  – Case 353-10, 43 year old male, venous blood
    • Found dead in the basement of a house
    • Suffered from diabetes
    • No medications but heavy smoking and alcohol abuse
FbF data extraction

Find by Formula Extraction (FbF):
- Grouping of different ion species
- Adding MS/MS spectra
  \(\rightarrow\) to one “Compound”!

Data courtesy of
Sebastian Broecker and Fritz Pragst
Institute of Legal Medicine, University Hospital Charité
Turmstraße 21, Building N, 10559 Berlin, Germany
Example

MS Database Search

- Isomers can’t be distinguished,
- Accurate mass and isotopic pattern allows for empirical formula confirmation
Example

MS/MS Library search

- Isomers are identified
- MSMS spectra containing structural information

- Benzoylecgonine?
- Roletamide?
- Norcocaine?

- Dexamisole?
- Tetramisole?

- Cocaine?
- Fenoterol?
- Hydromorphinol?
- Scopolamine?

Measured

Comparison

Library
Qualitative screening using All Ions MS/MS

What is new about All Ions MS/MS?

• **All Ions MS/MS offers a complete workflow for screening, verification and setup of a quantitation method**

• **It features:**
  – Support of TOF as well as QTOF
  – Easy setup of the acquisition method
  – **Unique** identification of product ions using Agilent’s MS/MS libraries
  – **Unique** chromatographic confirmation of product ions using Agilent’s unique co-elution score
  – Auto-dynamic setup of Quant method based on the identification results

• **Additional possibility to:**
  – Re-interrogate data for unknowns without reacquiring
  – Quickly add unknowns to the MS/MS library to include them in the forensic screening
**ALL Ions MSMS Workflow**

- Modified Find by Formula Algorithm for All ions
- Use PCDL Lib as source of fragments
- Export modified CEF file for Quant method building
- Quant requirement: Ability to convert new CEF file in Quant method and Quantitate on All Ions data file.
- PCDL Requirement: PCDL library content for pesticide available.
Step 1: Fragmentation without Isolation on TOF or Q-TOF

a) First Scan: Low fragmentation energy to analyze precursors

b) Second Scan: High fragmentation energy to analyze fragment ions

Low energy spectrum

High energy spectrum

July 25th, 2013
Forensic Seminar All Ions MS/MS
Untargeted Drug Screening in Postmortem Blood
All Ions MS/MS Automated Workflow

Step 2: Find by Formula Extracts Precursors using PCDL database

Qualify Precursor Match with Accurate Mass and Isotopic Distribution
Step 3: Extract Corresponding Fragment Ions

Cpd 11: zopiclone: +ESI HighE Scan (4.862-5.023 min, 20 Scans) O4406_alpra+meth.d...

Fragments from PCDL

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

Accurate Mass MS/MS Library for Thousands of Forensic Compounds
Untargeted Drug Screening in Postmortem Blood
All Ions MS/MS Automated Workflow

Step 4: Precursor and Fragment Correlation/Coelution

Overlaid Precursor and Fragment Ion Chromatograms

Coelution Plot

Agilent’s UNIQUE Coelution Score Enables Confident Identification
## Untargeted Drug Screening in Postmortem Blood

### LC-Method

<table>
<thead>
<tr>
<th>Agilent UHPLC 1290 System</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Column</strong></td>
</tr>
</tbody>
</table>
| **Mobile phase** | A= 10mM ammonium formate + 0.05% formic acid in water  
B= 0.05% formic acid in acetonitrile |
| **Flow rate** | 0.5 mL /min |
| **Gradient program** | Time (min) | B (%) |
| | 0 – 0.6 | 1 |
| | 0.7 | 5 |
| | 8 | 50 |
| | 10 – 11 | 95 |
| **Stop time** | 12 |
### Untargeted Drug Screening in Postmortem Blood

**MS-Method**

<table>
<thead>
<tr>
<th>Ion source</th>
<th>AJS ESI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polarity</td>
<td>Positive/Negative</td>
</tr>
<tr>
<td>Ion spray voltage</td>
<td>3500 V (Pos) / -3000 V (Neg)</td>
</tr>
<tr>
<td>Dry gas temperature</td>
<td>300°C</td>
</tr>
<tr>
<td>Dry gas</td>
<td>6 L/min (N₂)</td>
</tr>
<tr>
<td>Nebulizer pressure</td>
<td>35 psi (N₂)</td>
</tr>
<tr>
<td>Sheath Gas Flow</td>
<td>10 L/min (N₂)</td>
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<tr>
<td>Sheath Gas Temp</td>
<td>375°C</td>
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</table>

**MS parameters (MS mode)**

<table>
<thead>
<tr>
<th>Scan range</th>
<th>50 – 1000 amu</th>
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</thead>
<tbody>
<tr>
<td>Scan rate</td>
<td>1.5 spectra/sec</td>
</tr>
</tbody>
</table>

**All Ions MS/MS mode**

<table>
<thead>
<tr>
<th>Three segments at 0 eV, 20 eV, and 40 eV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scan range</td>
</tr>
<tr>
<td>Scan rate</td>
</tr>
</tbody>
</table>

Automatic internal reference mass correction was applied using purine \( m/z 121.0509 \) and HP921 \( m/z 922.0098 \).
Untargeted Drug Screening in Postmortem Blood
Differentiation and Identification of Isomers

Worst Case Scenario: Coeluting Isomers at Different Concentrations

Alprazolam

Methadone

Coeluting with ~50x Concentration Difference
Untargeted Drug Screening in Postmortem Blood
Differentiation and Identification of Isomers

Similar precursor \( m/z \) values and overlapping isotopic clusters

Worst Case Scenario: Overlapping Isotopic Distribution
Untargeted Drug Screening in Postmortem Blood
Compound Confirmation-Methadone

Good Isotopic pattern and abundance match

Confirmation with seven qualified fragments

Confident ID for High Concentration Coeluting Isomer
Untargeted Drug Screening in Postmortem Blood
Compound Confirmation-Alprazolam

Good isotopic pattern match-despite low concentration

Alprazolam confirmed with two qualified fragments

AND Confident ID for Low Concentration Coeluting Isomer
The All Ions MS/MS advantage for forensic
Real case example (Forensic Lab, Sweden)

EDDP product ion spectra (Literature)
High Productivity: Compound Details View

[Image of a software interface showing a compound list with various details such as compound name, molecular formula, retention time, and mass accuracy.]

- **Compound List**
  - [Sample Details]
  - [Mass Spectra]
  - [Identification Results]

The Measure of Confidence

For Forensic Use.

Agilent Technologies

Forensic Seminar All Ions MS/MS
July, 25th, 2013
Automatic Creation of Quantitation Method

Compound results
- Major adducts
- Accurate mass EICs for quantifier and qualifier
- Relative responses
- Retention times

Quantitation method
- Automatic selection of quantifier and qualifier(s)
- Reporting of qualifier ratios
- Isotope pattern comparison and accurate mass metrics as QC
Quant Batch at a Glance
All Ions Method imported into quant

Counts

199.0754, 171.0804, 317.1859
Ratio = 31.4 (100.0 %)
Ratio = 8.8 (100.0 %)
Ratio = 24.7 (100.0 %)

+ Scan (3.144-3.251 min, 8 scans) 3CEs_001.d
Mass Match Score=99.2 (M+Na+)
Counts

300.1595

317.1858
322.1414 (M+Na+)

Mass-to-Charge (m/z)

Acquisition Time (min)

Counts

30.0
30.5
31.0
31.5
32.0
32.5

0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
1.2
1.3
1.4
1.5
1.6
1.7
1.8
1.9
2.0
2.1
2.2
2.3
2.4
2.5
2.6
2.7
2.8
2.9
3.0
3.1
3.2
3.3
3.4
3.5
3.6
3.7
3.8
3.9
4.0

The Measure of Confidence

For Forensic Use.
All Ions MS/MS evaluation
Based on MS validation – see reference

<table>
<thead>
<tr>
<th>Formula</th>
<th>RT</th>
<th>Mass</th>
<th>Name</th>
<th>Coelution score</th>
<th>Score (Tgt)</th>
<th>Area</th>
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<tbody>
<tr>
<td>C18H22N25</td>
<td>7.79</td>
<td>298.1504</td>
<td>allopamezine</td>
<td>3 (5)</td>
<td>80.57</td>
<td>1710240</td>
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<td>C17H23C4N4</td>
<td>8.12</td>
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<td>506976</td>
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<td>C20H23N4</td>
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<td>277.1830</td>
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<td>82.18</td>
<td>2373320</td>
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<tr>
<td>C15H12N2O</td>
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<td>236.0950</td>
<td>carbamazepine</td>
<td>4 (5)</td>
<td>89.66</td>
<td>587865</td>
</tr>
<tr>
<td>C22H14F2N2O</td>
<td>6.80</td>
<td>324.1638</td>
<td>chloralpropamidine</td>
<td>5 (5)</td>
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<td>2330169</td>
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<tr>
<td>C19H18C3N2</td>
<td>8.55</td>
<td>314.1560</td>
<td>clopromazine</td>
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**References**

Identification of isomers
Clobazam and Temazepam, C₁₆H₁₃ClN₂O₂

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Identification of isomers
Norclobazam and Oxazepam, $C_{15}H_{11}ClN_2O_2$

For Forensic Use.
Identification of isomers
Norclobazam and Oxazepam, C$_{15}$H$_{11}$ClN$_2$O$_2$

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Searching against the 7500 compound database

For Forensic Use.
Searching against the 7500 compound database

The Measure of Confidence

For Forensic Use.
Summary

All Ions MS/MS extends the use of high res mass spec in forensic

- Data dependent as well as data-independent screening possible
- New forensic tox library allows for unambiguous identification and easy method setup (More than 9000 compounds with 3000 comp. w. spectra)

All Ions MS/MS Increases Confidence in ID

- Simple Method Setup
- Automated data processing
- Accurate Mass MS/MS Library Extraction
- Coelution Plot with Coelution Score

Reanalyze data any time, without reinjection of samples

Review data quickly, increasing productivity

Seamless integration of compound identification and quantitation for easy and quick batch review

Accurate quantitation of drugs in real samples using UHPLC/Q-TOF technology
Thank you for your attention!

Any Questions?