What do we mean by “small”?

- MW < 1000 Dalton (not well defined)
- Analytes related to
  - Drug metabolism (pharmacokinetics)
  - Environmental science
  - Forensic science/Homeland security
  - Natural Products
  - Astrobiology model reactions
  - Ion-molecule reactions
- Small enough to produce “strange” results (e.g., involved in ion-molecule reactions)

“Big” biomolecules (enzymes) generate “small” organic molecules that trigger further biochemical/biological processes.
General Questions

• **Ionization method?**
  – Instrument availability
  – Sensitivity/Quantification
  – Time of analysis

• **“In vivo” or sample pre-treatment?**
  – Chromatographic/extraction methods: *the shorter, the better*
    • GC, HPLC, zip-tip, solid phase extraction (SPE), dialysis, etc.
  – Derivatization: *the simpler, the better*
    • to increase volatility (GC); to study neutral loss; to increase ionization efficiency

The Nitrogen Rule

• Compounds* that contain even number of N atoms have even nominal molecular weight

• Compounds* that contain odd number of N atoms have odd nominal molecular weight

But what about singly protonated molecules and accurate molecular weights??

* Common organic compounds
Ion Stabilities

- *Even electron ions are more stable than odd electron (radical) ions*

How about protonated molecules: even electron or not?

And how about ions formed by electron impact (EI) ionization?

Fragmentation of protonated molecules generated by protonation at lone pairs of heteroatoms.
Learning Check on Small Molecules Fragmentation

Screening and identification of unknown contaminants in water with liquid chromatography and quadrupole-orthogonal acceleration-time-of-flight tandem mass spectrometry

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\(^b\)Micromass Ltd., Father Road, Wythenshawe, Manchester M23 9LZ, UK
\(^c\)Micromass Europe, Transmissarrant 18, 1320 AC Almere, The Netherlands

### Accurate Mass Measurements (Micromass Q-TOF)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Elemental Composition</th>
<th>Retention Time (min)</th>
<th>Theoretical Mass</th>
<th>Measured Mass</th>
<th>Δ ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metribuzin</td>
<td>C₈H₁₅N₄O₅S</td>
<td>25.18</td>
<td>215.0967</td>
<td>215.0969</td>
<td>1.1</td>
</tr>
<tr>
<td>Pirimicarb</td>
<td>C₁₁H₁₉N₄O₂</td>
<td>11.43</td>
<td>239.1508</td>
<td>239.1501</td>
<td>-2.7</td>
</tr>
<tr>
<td>Diuron</td>
<td>C₉H₁₁Cl₂N₂O</td>
<td>30.20</td>
<td>233.0265</td>
<td>233.0260</td>
<td>-2.3</td>
</tr>
</tbody>
</table>

### Elemental Composition Hits (Search Results)

<table>
<thead>
<tr>
<th>Compound</th>
<th>[M+H]⁺ Elemental Composition</th>
<th>Calculated Elecomp Hits</th>
<th>NIST Search Hits</th>
<th>InfoSpec Search Hits</th>
<th>Total Structures Evaluated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metribuzin</td>
<td>C₈H₁₅N₄O₅S</td>
<td>9</td>
<td>4</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Pirimicarb</td>
<td>C₁₁H₁₉N₄O₂</td>
<td>9</td>
<td>14</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td>Diuron</td>
<td>C₉H₁₁Cl₂N₂O</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

Is this enough?  NO!  MS/MS and accurate masses of fragments needed!
Nominal Atomic Masses:
- H = 1
- C = 12
- N = 14
- O = 16

Where do you think protonation can occur?

Same chemical formula (exact mass) but Different Ion Structures!

Are they all *fragmenting* structures????

Predict two main fragments from the protonated structures above!
Accurate Mass Measurements on [M+H]^+ and selected fragments

<table>
<thead>
<tr>
<th>[M+H]^+ and fragments of Pirimicarb</th>
<th>Elemental Composition</th>
<th>Theoretical Mass</th>
<th>Experimental Mass</th>
<th>Δ ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>72</td>
<td>C₆H₆NO</td>
<td>72.0449</td>
<td>72.0465</td>
<td>21.7</td>
</tr>
<tr>
<td>182</td>
<td>C₁₀H₁₂N₂O</td>
<td>182.1293</td>
<td>182.1306</td>
<td>6.9</td>
</tr>
<tr>
<td>195</td>
<td>C₁₀H₁₉N₄</td>
<td>195.1610</td>
<td>195.1627</td>
<td>8.9</td>
</tr>
<tr>
<td>239</td>
<td>C₁₁H₁₉N₄O₂</td>
<td>239.1508</td>
<td>239.1501</td>
<td>-2.9</td>
</tr>
</tbody>
</table>

Fig. 2: Product ions spectra of pirimicarb, obtained at different collision energies (from top to bottom) 15, 20, 25 and 30 eV. For elemental composition of the fragments, see Table 4.

Circled ions - odd or even electron ions?
From which protonated form do you think the fragment at m/z 72 can be generated?

From which structures do you think fragment at m/z 72 can be formed?

<table>
<thead>
<tr>
<th>Compound</th>
<th>Formula</th>
<th>Compound</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pirimicarb-I</td>
<td>C_{11}H_{18}N_{4}O_{2}</td>
<td>Atrazine-IV</td>
<td>C_{16}H_{11}CN_{4}</td>
</tr>
<tr>
<td>Metribuzin-III</td>
<td>C_{6}H_{11}N_{4}O_{5}</td>
<td>Isoproturon-V</td>
<td>C_{16}H_{11}N_{3}O</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diuron-VI</td>
<td>C_{16}H_{10}Cl_{2}N_{2}O</td>
</tr>
</tbody>
</table>

m/z 72 C_{3}H_{6}NO
Predict structures for fragments at m/z 114 and 83
(hint: use Nitrogen Rule)

\([M+H]^+ : \text{C}_{13}\text{H}_{26}\text{N}\)

Predict Compound Based on MS/MS spectra

\([M+H]^+ : \text{C}_{15}\text{H}_{13}\text{N}_2\text{O}\)

Fig. 4. Product ion mass spectra of an unknown compound with retention time 23.6 min found in surface water extract and the two most plausible structures. Collision energy: 15 eV (A) and 30 eV (B).
Select the Precursor Ion Based on MS/MS Spectra on Next Page

Fig. 6. Possible structural hits for the elemental compositions calculated for the determined exact mass (279.9971 Da) of an unknown compound with retention time 28.3 min, present in surface water extract.

Fig. 7. Product ion spectra of an unknown compound with retention time 28.3 min, obtained at different collision energies (from top to bottom: 15, 20, 25 and 30 eV.)
Take Home Messages

- Accurate masses reduce # of possible structures but, generally, not exclusive
- MS/MS is necessary
- Fragmentation rules (e.g., nitrogen rule, odd/even electron ions) apply but MS/MS spectra instrument and collision energy dependent
- Own database is useful

Marc A. LeBeau, M.S.; Madeline A. Montgomery, B.S.; Jurand R. Wagner, B.S.; and Mark L. Miller, Ph.D.

Analysis of Biofluids for Flunitrazepam and Metabolites by Electrospray Liquid Chromatography/Mass Spectrometry
J. Forensic Sciences, 2000, 45, 1133-1141

Sedative effects Facilitate sexual assaults
SPECIAL FEATURE: PERSPECTIVE

Strategy for structural elucidation of drugs and drug metabolites using (MS)<sup>n</sup> fragmentation in an electrospray ion trap

Zenzaburo Tozuka,*, Hayato Kaneko, Toshifumi Shiraga, Yasuyuki Mitani, Manabu Bappu, Shigeyuki Terasita, Akio Kawamura and Akira Kagayama

*Biopharmaceutical and Pharmacokinetics Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan
a. Measure the mass spectra of unchanged drug and its metabolites.
b. Prepare a table comparing $(MS)^n$ fragment ions of a drug with those of its metabolites.
c. Compare the spectral patterns of the drug before and after metabolism.
d. Assign the characteristic peaks of each metabolite in the mass spectrum.
e. Elucidate the structures of the metabolites.

Figure 3. CID mass spectra of the protonated molecules of tiaramide (top) and the single stage mass spectrum before the CID experiment showing the parent ion selected as the dominant peak at m/z 356 (bottom).
(MS)² fragmentation pathways of protonated tiaramide structures.

Metabolites of tiaramide and their ESI ion trap MS

Tiramide → Tiaramide aldehyde → Tiaramide carboxylic acid

Tiramide aminal

Tiramide-N-oxide → Dehydroxyethyltiaramide

Metabolism of tiaramide in humans
Integration of Knowledge-Based Metabolic Predictions with Liquid Chromatography Data-Dependent Tandem Mass Spectrometry for Drug Metabolism Studies: Application to Studies on the Biotransformation of Indinavir

M. Reza Anari,* Rosa I. Sanchez, Ray Bakhtiar, Ronald B. Franklin, and Thomas A. Baille

Department of Drug Metabolism, Merck Research Laboratories, WP7SA-203, Summitown Pk., West Point, Pennsylvania 19486

**Figure 1.** Metabolite identification strategy based on integration of knowledge-based metabolic predictions with liquid chromatography list-dependent tandem mass spectrometry.
Product ion spectra of protonated indinavir; (A) MS² product ion spectrum of m/z 614 and (B) MS³ product ion spectrum of m/z 364.

Knowledge-based metabolic prediction of indinavir
The Trinity of “Omics”

Genomics – Proteomics - Metabolomics

Applications of metabolomics in cancer research
Kathleen A. Vermeersch and Mark P. Styczynski
J. Carcinogenesis, 2013, 12, doi: 10.4103/1477-3163.113622
INNOVATION Metabolomics: the apogee of the omics trilogy
Gary J. Patti, Oscar Yanes and Gary Sluzdak
Molecular Cell Biology, 2012, 13, 263-269
Anal. Chem. 2006, 78, 779-787

XCMS: Processing Mass Spectrometry Data for Metabolite Profiling Using Nonlinear Peak Alignment, Matching, and Identification
Colin A. Smith, Elizabeth J. Wand, Grace O’Malley, Ruben Akkayy, and Gary Siuzdak
Anal. Chem. 2006, 78, 779-787
Progenesis QI
http://www.nonlinear.com/progenesis/qi/how-it-works/

Anal. Chem. 2014, 86, 9358-9361
Credentialed Features: A Platform to Benchmark and Optimize Untargeted Metabolomic Methods
Nathaniel Guy Mahieu, Xiaojing Huang, Ying-Jr Chen and Gary J. Patterson

Anal. Chem. 2014, 86, 9583-9589

MS/MS at different collision energies

A. Uracil

B. ADP

C. UDP-GlcA

D. Unknown of Mass 578.0993

E. Unknown of Mass 1169.3011

F. Unknown of Mass 848.7473