Identifying consumer product ingredients and their degradates with endocrine activity
An effects directed, non-targeted LC-MS/MS approach

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Endocrine Disruptors in Consumer Products
(active vs. disruptor)

Collapse of a fish population after exposure to a synthetic estrogen


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Comparative study of estrogenic potencies of estradiol, tamoxifen, bisphenol-A and resveratrol with two in vitro bioassays

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Endocrine disruptors in bottled mineral water: the E-Screen

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The Pesticides Endosulfan, Toxaphene, and Dieldrin Have Estrogenic Effects on Human Estrogen-Sensitive Cells

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Objectives

1. Broaden breadth of extractable compounds
2. Identify commonly detected compounds in sludge samples
   • LC/MS All-Ions acquisition
     • Targeted
     • Suspect
     • Non-Targeted
3. Correlation of molecular features with biological response
   • Prioritize features using CALUX bioassays
     • Estrogen (ER) activity
     • Aryl Hydrocarbon Receptor (AhR) activity
     • Glucocorticoid (GR) activity
Workflow

Sludge Extraction

Agilent 6530 qTOF All-Ions Acquisition

Analysis

Target
Suspect
Non-Target

Fractionation

Estrogen Activity
Aryl Hydrocarbon Receptor Activity
Glucocorticoid Activity

Correlation Analysis
Experimental Design

• 14 1-gallon sludge samples collected from wastewater treatment facilities in CA
  • Sludge treatment:
    • Anaerobic digestion
    • Dewatering
      • Centrifugation
      • Polymer thickening
      • Belt press

1. Profile compounds frequently detected across samples
2. Identify endocrine active compounds present in multiple samples
Compounds of Interest

~50 compounds in literature, which:

1. Have been detected in sewage sludge
2. Are suspected endocrine disruptors

<table>
<thead>
<tr>
<th>Purpose</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hormones</td>
<td>Estradiol, Estrone, Norgestrel</td>
</tr>
<tr>
<td>Preservatives</td>
<td>2-phenylphenol, parabens</td>
</tr>
<tr>
<td>Plasticizers</td>
<td>BPA, Phthalates</td>
</tr>
<tr>
<td>Pesticides</td>
<td>Dichlorobenzene, DEET</td>
</tr>
<tr>
<td>Fungicides</td>
<td>Miconazole, chlotrimizole</td>
</tr>
<tr>
<td>Fragrances</td>
<td>Tonalide, Galaxolide, musk ketone</td>
</tr>
<tr>
<td>Antimicrobials</td>
<td>Triclosan, Triclocarban</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>Trimethoprim, Sulfamethoxazole, Warfarin</td>
</tr>
<tr>
<td>Cosmetics</td>
<td>Benzophenone</td>
</tr>
<tr>
<td>Detergents</td>
<td>Octylphenol</td>
</tr>
</tbody>
</table>

Chose subset of this list for method optimization
Analytical Method

- Agilent 6530 qTOF
- *All-ions* Data Independent Acquisition
  - Collision Cell: 0, 10, 40eV
- Agilent Zorbax Eclipse C18 (2.1 x 100mm, 1.8uM)
Extraction of Endocrine Active Compounds from Sewage Sludge: pesticides, antibiotics, fragrances, preservatives, hormones, antimicrobials, detergents, etc.

- pH 2 and pH 7 fractions
- Sonication with 1:1 ACN:MeOH
- Agilent Bond Elut Plexa SPE
  - Washes-
    - 10% MeOH in H₂O
    - H₂O
  - Elution with 5% MTBE in MeOH
Sludge Extraction

Spike-Recovery

Matrix Factor = \frac{500 \text{ ppb standard}}{500 \text{ ppb matrix spike}}

Matrix Factor

<table>
<thead>
<tr>
<th>Compound</th>
<th>LOD (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-phenylphenol</td>
<td>0.5</td>
</tr>
<tr>
<td>4-tert-octylphenol</td>
<td>1</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>0.25</td>
</tr>
<tr>
<td>Estriol</td>
<td>1</td>
</tr>
<tr>
<td>Estrone</td>
<td>0.25</td>
</tr>
<tr>
<td>Ethynyl estradiol</td>
<td>0.5</td>
</tr>
<tr>
<td>Metoprolol</td>
<td>0.25</td>
</tr>
<tr>
<td>Miconazole</td>
<td>0.25</td>
</tr>
<tr>
<td>Norgestrel</td>
<td>0.25</td>
</tr>
<tr>
<td>Sulfamethoxazole</td>
<td>0.25</td>
</tr>
<tr>
<td>Triclocarban</td>
<td>10</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>0.25</td>
</tr>
</tbody>
</table>
Sludge Extraction

% Recovery

Positive Ionization

Negative Ionization

TRIMETHOPRIM
METOPROLOL
SULFAMETHOXAZOLE
CARBAMAZEPINE
MICONAZOLE
NORGESTREL
TRICLOCARBAZ
ESTRADIOL
ETHINYL ESTRADIOL
ESTRONE
2-PHENYLPHENOL
4-TERT-OCTYLPHENOL

UCDUMMY
Data Evaluation

1. Target Screen
   • List of compounds from literature

2. Suspect screen
   • Agilent Personal Compound Database Libraries
     • Water Contaminants
     • Forensic Toxicants
     • Pesticides

3. Non-Target Screen
   • Recursive Molecular Feature Extraction
   • Molecular Formula Generator
   • Confirm with reference standards, spectral libraries etc.
Suspect Screening

Tentative Identification –
• Within 15ppm mass accuracy of library
• Confirmed with at least 1 fragment ion
• Found in 3/4 extraction replicates
• >70% Score

Tentative Suspect Hits

<table>
<thead>
<tr>
<th></th>
<th>Water Contaminants</th>
<th>Forensic Toxicants</th>
<th>Pesticides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 9</td>
<td>6</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>Sample 10</td>
<td>15</td>
<td>41</td>
<td>10</td>
</tr>
<tr>
<td>Sample 11</td>
<td>2</td>
<td>5</td>
<td>1</td>
</tr>
</tbody>
</table>

![Agilent Library Hits table and graphs]
Non-Target Screen

- Recursive Molecular Feature Extraction
  (Agilent Profiler (B.08))
  - RT ± 0.2 min
  - Mass ± 10ppm
- Identify high priority features
  (Agilent Mass Profiler Professional (12.6.1))
## Non-Target QC

<table>
<thead>
<tr>
<th>Compound</th>
<th>m/z</th>
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<tbody>
<tr>
<td>Carbamazepine</td>
<td>237.102255</td>
</tr>
<tr>
<td>DEET</td>
<td>192.1383142</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>296.023993</td>
</tr>
<tr>
<td>Efavirenz*</td>
<td>316.0346907</td>
</tr>
<tr>
<td>Erythromycin*</td>
<td>734.93408</td>
</tr>
<tr>
<td>Flunixin</td>
<td>297.0845621</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>310.1413487</td>
</tr>
<tr>
<td>Fluvoxamine</td>
<td>319.1628124</td>
</tr>
<tr>
<td>Lamotrigine</td>
<td>256.0175</td>
</tr>
<tr>
<td>Mefenamic acid</td>
<td>242.1175787</td>
</tr>
<tr>
<td>Methyl Dihydrojasmonate*</td>
<td>227.1641946</td>
</tr>
<tr>
<td>Metoprolol</td>
<td>268.190741</td>
</tr>
<tr>
<td>Miconazole</td>
<td>414.993323</td>
</tr>
<tr>
<td>Norgestrel</td>
<td>313.216223</td>
</tr>
<tr>
<td>Sulfamethoxazole</td>
<td>254.0604</td>
</tr>
<tr>
<td>Tonalide*</td>
<td>259.205664</td>
</tr>
<tr>
<td>Triclocarban</td>
<td>314.98535</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>291.145178</td>
</tr>
</tbody>
</table>
Feature Extraction Results

- ± 20ppm mass accuracy
- Present in 75% of replicates

<table>
<thead>
<tr>
<th>Sample</th>
<th>POS</th>
<th>NEG</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 9</td>
<td>8504</td>
<td>1853</td>
<td>10,357</td>
</tr>
<tr>
<td>Sample 10</td>
<td>4915</td>
<td>2257</td>
<td>7,172</td>
</tr>
<tr>
<td>Sample 11</td>
<td>9061</td>
<td>3641</td>
<td>12,702</td>
</tr>
</tbody>
</table>
Non-Target Screen

- Prioritizing entities
  - Detection Frequency
    1. Ubiquitous use in consumer products
    2. Environmentally persistent
    3. Recalcitrant in sludge

One-way ANOVA
P-value cutoff < 0.05
Fold Change of 2 against blank

3990 --> 224 entities
224 Entities

tMSMS

Molecular Structure Correlator (Agilent B.07)

Spectra 0eV & 40eV

Triclosan m/z

Fragment 1 m/z
### Tentatively Identified Compounds

<table>
<thead>
<tr>
<th>Tentatively Identified Compounds</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>(14E)-16-Pentyloxyacyclocyclohexadec-14-en-12-yn-2-one</td>
<td>3-[Bis(6-aminoethyl)amino]tetrahydro-4H-thiopyran-4-one</td>
</tr>
<tr>
<td>(2S)-2-Acetamido-6-amino-N-[(2S)-6-amino-1-hydrazino-1-oxo-2-hexanyl]hexanamide</td>
<td>6,6'-[1,2-Propanediylbis(iminomethyllylidene)]bis(4-dodecyl-2,4-cyclohexadien-1-one)</td>
</tr>
<tr>
<td>(5S)-1-(Diaminomethylene)-2-hydroxy-5-[(2S)-2-hydroxynonyl]pyrroloidinium</td>
<td>cladroic acid</td>
</tr>
<tr>
<td>(8S)-1,13-Bis[(2S)-2-methylbutanoyl]-8-[(2Z)-2-undecen-1-yl]-1,5,9,13-tetraazacyclooctadecan-6-one</td>
<td>L-Lysine, L-lysyl-N⁵-(diaminomethylene)-L-ornithyl-N⁵-(diaminomethylene)-L-ornithyl-L-phenylalanyl-L-lysyl</td>
</tr>
<tr>
<td>10-{{[2E]-2-(Hydroxyimino)acetyl]amino}-N,N-dimethyl-1-decanaminium</td>
<td>L-Serine, L-α-aspartyl-L-asparaginyl-L-α-aspartyl-L-lysyl-N⁵-(diaminomethylene)-L-ornithyl-L-prolyl-</td>
</tr>
<tr>
<td>2-{{6-[Diaminomethylene]amino}hexyl]amino}-2-oxoethyl (4-aminobutyl)carbamate</td>
<td>N-(1,4-Dioxan-2-ylmethyl)-N-methyl-6-(4-thiomorpholinyl)[1,2,5]oxadiazolo[3,4-b]pyrazin-5-amine</td>
</tr>
<tr>
<td>2-Isobutylundecyl 1,3-thiazole-5-carboxylate</td>
<td>N-[1-Cyclohexyl-3-(ethylamino)-2-propanyl]-3-[(3-methoxypropoxy)(phenyl)methyl]-1-piperidinecarboxamide</td>
</tr>
<tr>
<td>3-[2-(4-Amino-6-hydroxy-2-pyrimidinyl)ethyl]-1-{2-methoxyethyl}-1(1,3-thiazol-2-ylmethyl)urea</td>
<td>N-[1-Cyclohexyl-3-(methylamino)-2-propanyl]-3-[(3-ethoxypropoxy)(phenyl)methyl]-1-piperidinecarboxamide</td>
</tr>
<tr>
<td>triclosan</td>
<td>N²-Acetyl-N-[[3-(1-methyl-1H-pyrazol-4-yl)-1,2,4-oxadiazol-5-yl]methyl]methioninamide</td>
</tr>
</tbody>
</table>
Non-Target Screen

- Prioritizing entities
  - Detection Frequency
- Effects-Directed Analysis
  - Biological response
    - Estrogenic
    - Aryl Hydrocarbon
    - Glucocorticoid

Peak Prioritization via CALUX bioassays

- Measures ligand-receptor binding
  - Estrogen
  - Aryl hydrocarbon
- Ligand-receptor binding induces transcription of firefly luciferase
  - Luciferin/Luciferase degradation
- Measures both antagonist and agonist properties

Effect-Directed Correlation Analysis

<table>
<thead>
<tr>
<th></th>
<th>+ control</th>
<th>- control</th>
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</thead>
<tbody>
<tr>
<td>998</td>
<td>8033</td>
<td>1877</td>
</tr>
<tr>
<td>1239</td>
<td>7994</td>
<td>2450</td>
</tr>
<tr>
<td>914</td>
<td>11892</td>
<td>2229</td>
</tr>
<tr>
<td>696</td>
<td>521</td>
<td>1425</td>
</tr>
<tr>
<td>914</td>
<td>5918</td>
<td>6816</td>
</tr>
<tr>
<td>696</td>
<td>6141</td>
<td></td>
</tr>
<tr>
<td>555</td>
<td>1522</td>
<td>1421</td>
</tr>
<tr>
<td>1225</td>
<td>1331</td>
<td>1314</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Androgenic Response (T47D.ARE cell line)

• HPLC fractionation (Agilent Zorbax Eclipse C18) of 125 ppb analytical standard
• Fraction 3 = 8.8 – 12.7 minutes (57 - 76% B (acetonitrile + 0.1% FA))
Fraction 3
RT 8.8 – 12.2 min
7/26 compounds

Carbamazepine [M+H] 237.1023
RT 9.445

DEET [M+H] 192.1383
RT 10.398

Flunixin [M+H] 297.0846
RT 11.004

Fluvoxamine [M+H] 319.1628
RT 9.150

Fluoxetine [M+H] 310.1413
RT 9.549

Miconazole [M+H] 414.9933
RT 11.056

Norgestrel [M+H] 313.2162
RT 11.992
Next Steps

1. Identify & profile commonly detected compounds in sludge
2. Measure ER, AhR, & GR activity
   - Measure activity of fractions
3. Correlate compounds identified in sludge to endocrine activity
Acknowledgements

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