Addressing Sample Stability Concerns in Large-Scale LC-MS Metabolomics Studies

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Discovery Themes
The Ohio State University
The OSU Discovery Themes Initiative (discovery.osu.edu)

Institution-wide strategic planning effort to enhance excellence in a number of key research areas, as accomplished through faculty hiring, student training opportunities, and industry partnerships.

- Brain Injury
- Data Analytics
- Foods for Health (Food and Nutritional Metabolomics for Personalized Health)
- Food and AgriCultural Transformation
- Humanities and the Arts
- Infectious Diseases
- Materials and Manufacturing for Sustainability
- Sustainable and Resilient Economy
What Will the Food and Nutritional Metabolomics Initiative Discover?

- A better understanding of individual biochemical variability in response to diet- and food-based interventions through:
  - Identifying **metabolic signatures** of individuals and diet
  - Discovering **new molecular biomarkers** that define healthy states and disease subtypes
  - Developing scientifically **sound dietary recommendations** and public health messages for individuals and specific groups
  - Understanding factors related to health maintenance and disease prevention
Outline

- Exposomics and human variability
- Sources of analytical variability in large clinical studies
- Post-extraction stability of urine and plasma
- Instrument stability across large analytical blocks of urine samples
Centers for Disease Control-
“The exposome can be defined as the measure of all the exposures of an individual in a lifetime and how those exposures relate to health.”
Human variability can be great

- Humans are unique in their metabolomes due to genetics, microbiota, age, lifestyle, etc.
- Differences in how people metabolize drugs, dietary components and other compounds from the environment
- Can make it challenging to assess exposure
- Leads to heterogeneity in biological response
- Metabolomes are dynamic and variable
Human variability can be great

**Nicotine Metabolism**

- 70-80% of nicotine is metabolized to cotinine
- Considerable interindividual differences in the rate of nicotine metabolism have been observed
- Differences in metabolism might help explain/predict nicotine dependence and smoking behavior

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Human variability can be great

**Isoflavone Metabolism**

- Isoflavones in the diet come primarily from soybeans and are metabolized by the colonic microflora.
- Study with soy bread showed significant differences in isoflavone metabolism with the most variety in daidzein metabolism.
- 4 major metabolotypes were identified through clustering analysis with people varying in types and levels of metabolites produced.

Challenges with large clinical metabolomics studies

- Consistency and standardization are key in metabolomics studies
  - The larger clinical studies become, the harder it can be to limit variability

- Sources of variability include-
  - Sample collection
  - Sample handling/storage
  - Sample prep and data acquisition
Variability in clinical metabolomics studies

Sample Collection

- Simple and non-invasive collection procedures are preferred
- Challenges exist with multi-site studies
- Need to consider how samples are collected, when samples are collected, materials used in the collection process
- Pilot studies can be used to help identify issues in the sample collection process
Sample Collection
Example 1

Plasma Metabolomics

- Random selection of samples were pulled from a large clinical study on smoking (PI: Peter Shields)
- Pilot metabolomics experiment conducted to evaluate the extraction and LC-MS method for plasma
- Replicates and pooled QCs were included to assess reproducibility
- Extracts were analyzed by reversed phase UHPLC-QTOF-MS (ESI+/−)

Agilent 1290 Infinity UHPLC/6550 QTOF-MS
Sample Collection

Example 1

Metabolite database search for m/z 1226.5341
Sample Collection

Example 1

In-source fragments and adducts are consistent with diatrizoate.

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Sample Collection

Example 1

Diatrizoate

<table>
<thead>
<tr>
<th>Metabolite Identification</th>
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<tbody>
<tr>
<td>Common Name</td>
<td>Diatrizoate</td>
</tr>
<tr>
<td>Description</td>
<td>Diatrizoate is only found in individuals that have used or taken this drug. It is a commonly used x-ray contrast medium. As diatrizoate meglumine and as Diatrizoate sodium, it is used for gastrointestinal studies, angiography, and urography. (PubChem)Diatrizoate is an iodine-containing X-ray contrast agent. Isolated contrast agents were among the first contrast agents developed. Iodine is known to be particular electron-dense and to effectively scatter or stop X-rays. A good contrast agent requires a high density of electron-dense atoms. Therefore, the more iodine, the more &quot;dense&quot; the x-ray effect. Iodine based contrast media are water soluble and harmless to the body. These contrast agents are sold as clear colorless water solutions, the concentration is usually expressed as mg/mL. Modern iodinated contrast agents can be used almost anywhere in the body. Most often they are used intravenously, but for various purposes they can also be used intraarterially, intravenously (the spine) and intra-abdominally - just about any body cavity or potential space.</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Structure</th>
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<tr>
<td>HN</td>
<td></td>
</tr>
<tr>
<td>COO</td>
<td></td>
</tr>
<tr>
<td>H₂N</td>
<td></td>
</tr>
<tr>
<td>COO</td>
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</table>

<table>
<thead>
<tr>
<th>Synonyms</th>
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<tbody>
<tr>
<td>1. Amidotrizoate</td>
<td></td>
</tr>
<tr>
<td>2. Amidotrizoic Acid</td>
<td></td>
</tr>
<tr>
<td>3. Diatrizoate</td>
<td></td>
</tr>
<tr>
<td>4. Diatrizoate sodium salt</td>
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<tr>
<td>5. Diatrizoic acid</td>
<td></td>
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<tr>
<td>6. Diatrizoic acid sodium</td>
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<tr>
<td>7. Methalamic acid</td>
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<tr>
<td>8. Sodium amidotrizoate</td>
<td></td>
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<tr>
<td>9. Sodium diatrizoate</td>
<td></td>
</tr>
<tr>
<td>10. Urografin acid</td>
<td></td>
</tr>
<tr>
<td>11. Urogranic acid</td>
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<table>
<thead>
<tr>
<th>Chemical Formula</th>
<th>C₁₁H₁₂J₂N₂O₄</th>
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<td>Average Molecular Weight</td>
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<tr>
<td>Monoisotopic Molecular Weight</td>
<td>613.76637046</td>
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<td>IUPAC Name</td>
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<tr>
<td>Traditional IUPAC Name</td>
<td>diatrizoate</td>
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<td>CAS Registry Number</td>
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<td>SMILES</td>
<td>CCl(O=NC1=O)(C)(O)=O=C(C)(NC)(C)=O=CCl</td>
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<td>InChi Identifier</td>
<td>InChi:1S/C11H9I3N2O4a1-3c11(7-9-12(5(11-19)20)7(13)(10-8(9-14)16-4-18)18(1)1-2-13H.(H,15,17)H,16,18)H,19,20)</td>
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<tr>
<td>InChl Key</td>
<td>YVPYQUNQLQZFHG-UHFQAOYSA-N</td>
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</table>

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Sample Collection

Example 1

BD Vacutainer® CPT™

Cell Preparation Tube with Sodium Citrate

For the Separation of Mononuclear Cells from Whole Blood

Sterile Interior

Contains:

- 0.45 mL of 0.1 Molar Sodium Citrate Solution (Top Fluid Layer)
- 1.8 gm of Polyester Gel (Middle Layer)
- 1.0 mL of Polysaccharide/Sodium Diatrizoate Solution (FICOLL™ Hypaque™ solution, Bottom Fluid Layer)
- Silicone Coated Glass Tube
- Silicone Lubricated Rubber Stopper

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Sample Collection
Example 1

PCA colored by smoking status
Sample Collection
Example 1

PCA colored by smoking status

PCA colored by tube type

Tube type is driving separation

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Sample Collection
Example 2

Bronchoalveolar Lavage Fluid Metabolomics

- Samples collected from a clinical trial at OSU (PI: Peter Shields) to study the effects of electronic cigarettes on the lungs
- Untargeted metabolomics is being used to identify biomarkers related to e-cigarette usage
- Smoking related metabolites are of particular interest, in addition to endogenous metabolites
- Pilot metabolomics study has been conducted with BAL fluid to assess analytical protocol
  - Random subset of 24 samples
  - LC-MS (ESI +/-)
Sample Collection

Example 2

ESI+ BPC of bronchoaveolar lavage fluid extract

Mass spectrum of saturating peak

1\textsuperscript{st} and 2\textsuperscript{nd} isotopes are both saturating at a 5 \(\mu\)L injection

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Sample Collection

Example 2

Metabolite database search for m/z 235.1806
Sample Collection

Example 2

Metabolite database search for m/z 235.1806

Lidocaine is used as an anesthetic during the bronchoscopy procedure.
Lidocaine is important for comfort and cannot be eliminated from the bronchoscopy procedure. Must handle analytically. Can divert flow to waste between 4-4.5 min to protect the detector.
Variability in clinical metabolomics studies

Sample handling and storage

- Time to freeze
- Storage temperature
- Freeze-thaw cycles
Variability in clinical metabolomics studies

Sample handling and storage

- **Storage temperature**
  - Significant time- and temperature-dependent changes in plasma metabolites have been observed (see figure).
  - Plasma metabolites have been shown to be stable at -80 °C for at least 2.5 years (Pinto et al. Analyst. 2014, 139:1168-1177).

Copied from Moriya et al. Metabolomics. 2016, 12:179
Variability in clinical metabolomics studies

Sample handling and storage

• Freeze-thaw cycles
  – Should be avoided if possible, but at the very least should be consistent across samples
  – Noticeable changes by in the plasma metabolome by NMR have been reported after 3 freeze-thaw cycles (Pinto et al. Analyst. 2014, 139:1168-1177)
Variability in clinical metabolomics studies

Sample prep and data acquisition

- Multiple analysts
- Sample prep procedure
- Time to analysis following extraction (i.e. time in autosampler)
- Temperature of autosampler
- Instrument stability
  - Should be smallest source of variability
Stability of biological samples post-extraction

Urine Autosampler Study

Sample Prep
- Pooled urine
- 10x dilution w/ 0.1% FA
- Centrifuged 4°C for 10 min
- Aliquots into 50 HPLC vials

Data Acquisition
- Aliquots were loaded into autosampler at 4°C
- 10 conditioning samples injected first
- Waters BEH C18 Column, 12 min runs
- Run Continuously on Agilent 6550 QTOF (ESI+)

Data Pre-Processing
- Feature extraction and data reduction using Agilent Profinder and MPP
- Filtered to remove compounds present in <20% of injections
- Restricted ion abundance to 1000-900000

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Stability of biological samples post-extraction

Urine Autosampler Study

PCA of Pooled Urine Injections

Separation of pooled samples based on length of time in the autosampler

Injections cluster more closely toward end of batch

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Stability of biological samples post-extraction

Urine Autosampler Study

Mass vs. RT Plot of Compounds Significantly Different (P < 0.05) between Initial and Final Injections

- Observe both increasing and decreasing metabolites
- Many altered metabolites have small fold changes
- Significantly different metabolites seen throughout the run

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Stability of biological samples post-extraction

Urine Autosampler Study

Heat map of PCA component 1 loadings (organized by injection “group”)

Biggest changes appear to be in phospholipids (1.7-3.3 fold decreases)

Other changes include phenol, bilirubin derivatives, organic acids, and amino acid derivatives

Many of the increasing metabolites are unidentified
Stability of biological samples post-extraction

**Plasma Autosampler Study**

**Sample Prep**
- Pooled plasma
- MeOH extraction, centrifuged 4°C for 10 min
- Died down and reconstituted in H₂O
- Aliquoted into 50 HPLC vials

**Data Acquisition**
- Aliquots loaded into autosampler at 4°C
- 10 conditioning samples
- Agilent Eclipse Plus C18 column, 15 min runs
- Run continuously on Agilent 6550 QTOF (ESI+)

**Data Pre-Processing**
- Feature extraction and data reduction using Agilent Profinder and MPP
- Filtered to remove compounds present in <20% of injections
- Restricted ion abundance to 1500-1000000

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Stability of biological samples post-extraction

Plasma Autosampler Study

PCA of Pooled Plasma Injections

The time to injection explains more of the variation in the plasma data than the urine data (31% vs 11%)

Injections again cluster more closely toward end of batch

More conditioning or longer equilibration period?

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Stability of biological samples post-extraction

Plasma Autosampler Study

Mass vs. RT Plot of Compounds Significantly Different (P < 0.05) between Initial and Final Injections

Fold changes are generally small and greatest variation is seen at the end of the run

Increases in amino acids and peptide fragments

Some changes in polar lipids, lipid degradation products

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Continuous data acquisition and batch size limitations

- In LC-MS metabolomics experiments, running continuously is preferred
  - Avoid small shifts in retention time and instrument sensitivity
  - Minimize batch effects
- However, data quality can suffer if running continuously for too long
  - Loss of sensitivity, cleaning needed
  - Chromatography fails
Continuous data acquisition and batch size limitations

Urine Metabolomics Study

<table>
<thead>
<tr>
<th>Injection Order</th>
<th>Sample Type</th>
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<tr>
<td>1</td>
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<tr>
<td>2</td>
<td>Method blank</td>
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<tr>
<td>3-12</td>
<td>Conditioning QCs</td>
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<tr>
<td>13-20</td>
<td>Urine samples</td>
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<td>21</td>
<td>QC</td>
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<td>QC</td>
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<td>290</td>
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<td>291</td>
<td>Method blank</td>
</tr>
<tr>
<td>292</td>
<td>Solvent blank</td>
</tr>
</tbody>
</table>

- Samples from an OSU-Center of Excellence in Regulatory Tobacco Science project
- Urine collected from adolescents with self-reported cigarette and smokeless tobacco usage (PI: Peter Shields)
- Urine prep = centrifugation + dilution
- 278 urine samples including pooled QCs and random duplicates
- Agilent 1290 Infinity UHPLC/6550 QTOF-MS
- ESI+/-
- Reversed phase UHPLC
- H₂O 0.1% FA, ACN 0.1% FA
- Autosampler = 4°C
- Data processed using Agilent Profinder and MPP
Urine data should be normalized to handle significant differences in urine volume between subjects.

Normalization strategy used can greatly affect results from LC/MS metabolomics analyses. Various strategies have been evaluated by Warrack et al. J Chrom B. 2009, 877:547-552
Urine QCs cluster together in unsupervised multivariate analyses.

No significant differences in the individual batches of urine prepared throughout the continuous analytical block of 278.
Internal standards show little variability across study samples

Negative mode internal standard (4-nitrobenzoic acid) CV = 7.7%

Positive mode internal standard ($^{13}$C-labeled phenylalanine) CV = 7.3%

Internal standards can be used to monitor instrument performance run to run

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QCs can be used to assess consistency in the detection of various metabolites

![Chemical structures of nicotine, cotinine, and trans-3'-hydroxycotinine](image)

**3-Hydroxycotinine**

- Compound Intensity vs. QC Run Order
- CV = 8.4%
- Shaded area = 1 SD

**Cotinine**

- Compound Intensity vs. QC Run Order
- CV = 8.2%
- Shaded area = 1 SD

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Summary

- Pilot metabolomics studies are useful when working with new sets of samples
- How biological samples are collected is a significant source of variation
  - Blood collection tube type must be standardized
  - It is difficult to adapt human samples collected from non-metabolomics focused studies for metabolomics analyses
- Significant differences have been observed in pooled biological samples in the same analytical batch based on time to injection
  - Smaller prep batches are preferred with some equilibration time in the autosampler
- Internal standards and QCs show LC-MS stability in urine metabolomics study of 278 samples
  - Need to balance quality and feasibility
  - Larger analytical blocks become challenging from a sample prep perspective (in the absence of a competent robot!)
Acknowledgments

The James

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Ping-Ching Hsu – Assistant Professor (now at UAMS)

Nutrient and Phytochemical Analytics Shared Resource – CCC Core Lab
Ken Riedl – Acting Director, Senior Research Scientist

Personalized Food and Nutritional Metabolomics for Health
Discovery Themes at The Ohio State University
Learn more at discovery.osu.edu

BUCKEYE
Cruise for Cancer

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