A Robust LC-QQQ Metabolomics Platform for Polar Metabolite Profiling: Dynamic Biological Systems Meet Dynamic MRM

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Key points for today’s webinar:

• Metabolomic data provide a direct view of biochemical state

• We have developed a robust ion-paired reverse phase method and MS/MS transition library to enable analysis of polar metabolites
  – Retention time / transition libraries are critical for unambiguous compound identification. Database searches are insufficient

• Sample preparation and analysis to generate metabolomic data requires care and consistency, and is within the reach of many labs

• The real payoff of metabolomics is in understanding regulation of biological systems, not reinventing the wheel for analysis
What metabolomics means to my lab

Ion-paired reverse phase UPLC for analysis of polar metabolites

Our workflow from discovery to targeted analysis

Enabling biological discovery with targeted metabolomics
Metabolomics measures biochemical state – a fundamentally different view from genomics
With metabolomics, we’re looking at the painting, not asking which colors were on the artist’s table.

- **Genomics:** “What’s in the catalog?”
- **Transcriptomics / proteomics:** “What parts are in use?”
- **Metabolomics:** “What is the final product?”
Metabolism is ancient and well conserved enabling similar analysis of model organisms, bioprocess, and patients

C. Woese, Microbiol. Rev 1987
My group’s goal is to understand the wiring of the cell at transcription, protein, and metabolic levels.
• RNA measurement:
  • A,U,G,C chemistry, known template
  • Search space is known and limited

• Protein measurement:
  • Larger alphabet (20+) + modifications
  • Larger search space, still “knowable”

• Metabolite measurement
  • Vast chemical space
  • Large number of isomeric and isobaric compounds; separation is informative
Many metabolic reactions are conserved, enabling immediate cross-species analysis.
My group is focused on several specific areas within metabolomics:

- Small polar metabolites
- Analyses focusing on phosphosugars, organic acids, nucleic acids, and amino acids
- *Enzyme discovery and metabolic regulation*
Compounds of interest in metabolomics span a broad chemical space

- α-ketoglutarate
- sedoheptulose bisphosphate
- ergosterol
- Adenosyl-B12

Clasquin et al., Cell 2011

Watson et al., Cell 2014
Multiple, orthogonal techniques are needed to fully address the chemical diversity of metabolism.

- Metabolomics is a catch-all term, not a single tool.
- Many analytes are accessible by multiple techniques.
- My lab focuses on LC-MS, and uses CE-MS and NMR.
A snapshot of LC/MS systems in my group:

- Agilent 1290 UPLC pumps driving 1.8µm columns
- Two pumps, two columns per mass spectrometer
  - Offline regeneration for doubled throughput

- Full-scan “profiling” experiments
  - Excellent dynamic range
  - Compound ID via mass labeling, accurate mass, isotopologue resolution

- Inherently targeted assays
  - Hundreds of metabolic MRMs developed
  - Excellent sensitivity
  - Small data footprint, fast data analysis

(Bulletproof instrument)
- Mass-based fractionation
- In vitro reaction analysis

MassHunter Qualitative/Quantitative software platform
Single-point metabolomic analysis provides a measurement of levels, not reaction rates.
The “LC” in LC-MS is particularly important for the separation of related metabolites
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Jack of all trades, master of none

though oft times better than master of one

• Ion paired reverse phase methods provide moderate retention for many polar metabolites

• We find tributylamine (TBA) to be the optimal balance of lipophilicity and repeatable elution

• Peak shapes are good to excellent for many compounds
  – Less than 9 second average peak width
  – Even poorly shaped peaks are consistent, and can be reliably integrated using MassHunter Qual and Quant

• Ion-pairing methods should be used on dedicated instruments
Ion-paired reverse-phase separation provides retention of both polar and traditional RP analytes

- Many polar metabolites are poorly retained on ODS packings
- Addition of an ion-pairing agent provides additional functionality to stationary phase
- **Reverse-phase separation is maintained, expanding analyte range**
  - A: 97:3 (H₂O:MeOH) with 10mM TBA, 15mM Acetate
  - B: 100% MeOH
Ion-paired chromatographies are often plagued by retention time drift

- TBA aqueous-methanol binary system provides excellent retention and peak shape
  - *BUT* has progressive chromatographic drift over multiple injections
We use a ternary solvent system for reproducible ion-paired chromatography

- Column “stripping” with Acetonitrile, followed by re-loading / re-equilibration enables highly reproducible chromatography
  - Aqueous:MeOH is still used for analytical separation
  - *Note slight change in retention time (0.3’) compared to Aq:MeOH method*
  - *Retention times are consistent across thousands of injections*
An Agilent UPLC solution enables our high-throughput, highly reproducible ion-paired chromatography

- 1290 binary pump is connected to autosampler and to mass spectrometer
- Two identical columns are placed in column compartment
  - Agilent Extend C18 RRHD 2.1x150mm, 1.8µm
- 10-port, 2-position valve enables off-line column regeneration via second pump (1290 quaternary) which purges to waste
Our alternating column regeneration also performs an off-line backflush each cycle

- As the 1290 binary pump is driving an analytical gradient on Column #1, the quaternary pump is backflushing Column #2
- Moments before the next injection, the 10-port valve switches position
  - Column #2 is then connected to the binary pump and autosampler
Using two pumps enables the mass spectrometer to collect useful data at all times

- Use of alternating columns enables double the throughput per mass spectrometer

- A 1290 quaternary pump can be used in a single pump configuration (Aq:MeCN:MeOH), but with a 37 minute runtime
  - Use of a single pump also precludes per-cycle backflush
Our IP-RP method provides separation of a wide range of polar metabolites in 19 minutes

- Data for more than 100 representative transitions *in matrix* are shown
- Compound elution patterns are predictable based on chemical functionality
Highly consistent elution time enables tight dynamic MRM\textsc{s} and integration of difficult peaks

- We have optimized nearly 200 metabolites across a wide range of endogenous polar metabolites
- Several classes of peaks exhibit moderate tailing, but are still quantifiable

\begin{align*}
\text{Nicotinic Acid} & \quad \ln(y) = 7.328430 + 1.290416 \times \ln(x) - 0.087522 \times \ln(x)^2 \\
R^2 & = 0.99933740 \\
\text{Type: Second order ln, Origin: Ignore, Weight:1/x^2} \\
\end{align*}

\begin{align*}
\text{Hexosamine-phosphate} & \quad \ln(y) = 7.428932 + 1.008240 \times \ln(x) - 0.022267 \times \ln(x)^2 \\
R^2 & = 0.99996298 \\
\text{Type: Second order ln, Origin: Ignore, Weight:1/x} \\
\end{align*}
Retention time is an essential part of compound identification. MS/MS transitions and accurate mass are only part of the story.

- Metabolomic samples are inherently complex mixtures
- Many isobaric, isomeric, and chemically similar compounds present in cells
  - MRM transitions frequently involve common losses
- Database searches using spectra or accurate mass are ambiguous
  - A reference chromatography and matches to standardized RT enable confident identification

- Feature mis-annotation is a major problem without comparison to standards

Reproducible, standardized chromatography enables:
- Narrow dynamic MRM windows
- Confident metabolite identification
Consistent, standardized chromatography enables high-density MRMs to maximize acquisition efficiency

- Narrow retention time enables more MRM transitions per run
- Dynamic MRMs enable optimal use of instrument cycle time
  - Traditional “time segments” are inadequate to cover the wide range of elution times
Our ion-paired reverse phase method is reproducible across time and space
Analysis of large sample sets is possible due low retention time deviation across thousands of injections

62 different samples, 3 days

Aliquoted sample, 7 months of column use (>4000 injections)
Agilent’s Extend-C18 chemistry enables long-term chromatographic stability

- Extend-C18 columns are rated stable over a wide pH range
  - Other C18 packings have been plagued by gradual stationary phase voiding
- Little change in peak shape over thousands of injections
- Excellent column-to-column and lot-to-lot consistency
  - Data can be compared across large experimental campaigns (years!)
  - Integration of data from multiple different instruments
Our IP-RP chromatography enables rapid translation across instruments and platforms

- A representative biological sample was run on 6460 QqQ, 6550 qTOF
- EIC / MRM of metabolites retained across chromatogram shown
- Different columns, different solvent lots used
We leverage multiple instrumentation platforms for a complete metabolomics solution

- Compound-specific optimization of ESI source parameters
- Optimization of fragmentor energies
- Optimization of collision energies for transitions identified on qTOF platform
- Rapid analysis of hundreds of known metabolites

Shared ion-paired reverse-phase chromatography

- (novel metabolite identification)
- Accurate mass spectra of product ions across a wide range of collision energies
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Our workflow from discovery to targeted analysis

Enabling biological discovery with targeted metabolomics
• Our goal is to understand the metabolic phenotype of biological systems

• Compound identification and characterization are just the beginning

• We analyze many thousands of samples per year to study:
  – Mechanisms of metabolic regulation
  – Reaction kinetics
  – Linkage of genotype to metabolic phenotype
  – Dysregulation of metabolism in disease
Proper experimental design and sample prep are essential to generating interpretable results

- Samples and controls are grown, extracted, and analyzed in parallel
- Independent biological replicates are of greater value than technical replication
- Mock extraction / blank samples are necessary to exclude contamination from media or supplement components
- *Consistent sample collection and prep are essential to interpretable results*
Loading equal amounts of material is the first step to accurate metabolite quantitation

- Cell number and size is measured at the time of sample collection
  - Direct “Coulter” measurement
  - Flow cytometry
  - Optical density for microbes

- Total integrated cell volume is used to normalize during resuspension and sample loading
  - Cell number is a poor proxy, and should be used only if cells are demonstrated to not change size as a function of sample
We collect samples using a simple organic “quench and extract” approach

- Media is rapidly removed from cells
- Metabolism is rapidly quenched and extraction started by immersion in ice-cold (-20°C) MeCN:MeOH:H₂O
- Cells are freeze-thaw cycled to lyse cells and organelles
- Metabolite extract is separated from cells, dried under nitrogen, and resuspended in aqueous loading buffer
Place 25mm*1 µm nylon filter on cleaned manifold position

Mount sample chimney on filter. Visually inspect seal

Pipet sample into chimney (10mL max. vol per transfer)

Turn stopcock to begin apply vacuum and begin filtration

Immediately upon completion, transfer filter to 5mL microcentrifuge tube with pre-chilled extraction solvent

Ensure that filter is covered in solvent. Briefly vortex. Return to dry-ice or -80°C
Alternate protocol for extraction of adherent culture cells

Pre-cool aluminum block and extraction solvent on dry ice

Rapidly remove media by vacuum. Do not wash cells

Immediately add pre-chilled extraction solvent and place dish on aluminum plate

Triturate monolayer fully. Cells will detach, and be released into solvent

Transfer mixture of solvent and cells to a 5ml microcentrifuge tube. Keep on dry ice or at -80°C
Freeze-thaw sample for three cycles (-80°C to -20°C)

Cells are ruptured during repeated ice-crystal formation enabling consistent extraction

Note pelleted cell debris. This will be avoided when pipetting

Remove filter using clean forceps. Discard filter

Remove 80% of extract liquor, leaving pellet undisturbed.

Centrifuge at 12,000xg for 5’ (alternatively 15’ at 4,000xg) to clarify metabolite extract

Transfer extract to a fresh 1.5ml tube
Evaporate extraction solvent to dryness under pure nitrogen. Resuspend samples in UPLC water with spike-in standards.

Assemble vacuum manifold with a clean mass-spec grade polypropylene plate.

Mount filter plate to top of manifold, ensuring good seal. Transfer samples to filter plate and apply vacuum.

After filtration, sample will be collected in lower plate.

Transfer filtered sample to autosampler vial or seal plate for use in well-plate sampler.

If necessary, store filtered sample at -80°C until analysis.
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Disrupting traffic reveals the underlying roadmap

- Metabolic reactions are interconnected
- Not all connections are equal, and reactions are regulated
- Blocking metabolism can reveal:
  - Network connectivity
  - Relative usage of pathways
- Changes in steady-state levels upon disruption can be used to infer flux
Drug mechanism of action can be determined by analyzing metabolic traffic jams

- *E. coli* cultures were treated in replicate with various small molecule inhibitors
  - *Glyphosate*, a prototypical example, is shown here
- Targeted LC-MS analysis was used to measure intracellular metabolite levels
- Inhibited or altered reactions are apparent by change in product and substrate levels
Targeted metabolomic analysis can be used to identify altered reactions and disrupted pathways

- Several steps in the shikimate/chorismate pathway for aromatic amino acid biosynthesis are shown.

- Targeted LC-MS analysis of glyphosate-treated cells demonstrates:
  - Buildup of 3-phosphoshikimate and committed upstream metabolites
  - Depletion of chorismate and aromatic amino acids

- Inhibition of a single enzyme leads to coherent changes in up- and downstream metabolite levels.
Many disease states are “slowed traffic” rather than total gridlock. Targeted LC-MS provides quantitative measurements over a large dynamic range.

- Pathological accumulation of mevalonate is associated with several human diseases.
- Mevalonate kinase is an essential gene; disease alleles show reduced function.
- Quantitation of mevalonate levels in patient cell lines was performed using targeted LC-MS with spike-in reference.
- Despite our capacity to measure hundreds of metabolites, some questions require analysis of just one!
Different road conditions affect traffic: metabolism is dependent on growth context

- Cell lines representing a diverse panel of solid tumors were grown in monolayer tissue culture (in vitro) and as rodent xenograft (in vivo)
  - Columns on left and right panel are matched samples

- Cellular energy charge is drastically increased in vitro, as are levels of glycolytic intermediates

- Media components and their downstream metabolites are greatly increased in cultured cells

- The predominant metabolic signal is growth condition, not tissue of origin or clinical stage
Targeted LC-MS metabolomics is a powerful, accessible tool for measurement of biological systems

- Metabolomic data provide an orthogonal view of biology: direct biochemical phenotyping

- We have developed an ion-paired method that provides long-term chromatographic stability and is rapidly deployable in other labs

- Generating high-quality data begins with experimental design and sample prep

- Measuring levels of known metabolites provides excellent biological insight into a variety of research questions