

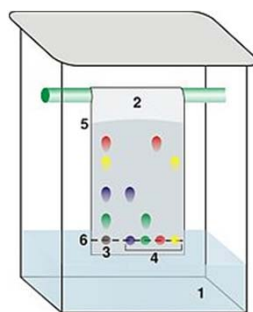
GBS 748

## LC-MS analysis of metabolites

Stephen Barnes, PhD  
4-7117; [sbarnes@uab.edu](mailto:sbarnes@uab.edu)

## Basis of Chromatography

- A moving (mobile) phase passes over an inert, stationary phase
  - The compounds differentially interact with the stationary phase and elute at different times



- The *stationary phase* can be paper, silica, coated silica and derivatized silica
- The *mobile phase* can be a gas or a liquid (organic solvent or water)

## LC-MS

- **The solid phase is typically silica or diatomaceous earth**
  - The silica surface can be made to be hydrophobic with attached alkyl groups (**reverse-phase**), or hydrophilic with attached  $\text{NH}_2$  groups (**normal phase**)
- **The mobile phase is a liquid**
  - Gradients of methanol, ethanol, isopropanol, or acetonitrile in water with volatile additives (0.1% formic acid or formic acid, 2-10 mM ammonium acetate or formate)
  - Trifluoroacetic acid is not used, nor are Tris or phosphate buffers

## Column phases

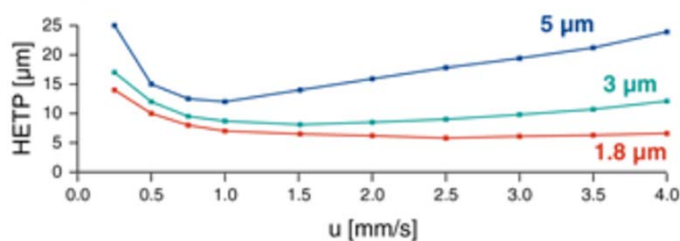
- **Reverse-phase**
  - $\text{C}_4$ ,  $\text{C}_5$ ,  $\text{C}_8$ ,  $\text{C}_{18}$ , phenyl-hexyl-bonded phases
- **Normal phase**
  - Bare silica, Cyano and amino-bonded phases
- **Hydrophilic interaction chromatography**
  - Bare silica, polyol-bonded phase
- **Particle sizes**
  - 5, 3, 2.5, 2.2  $\mu\text{m}$  and 1.7  $\mu\text{m}$  (for UPLC)

## Important equations in chromatography

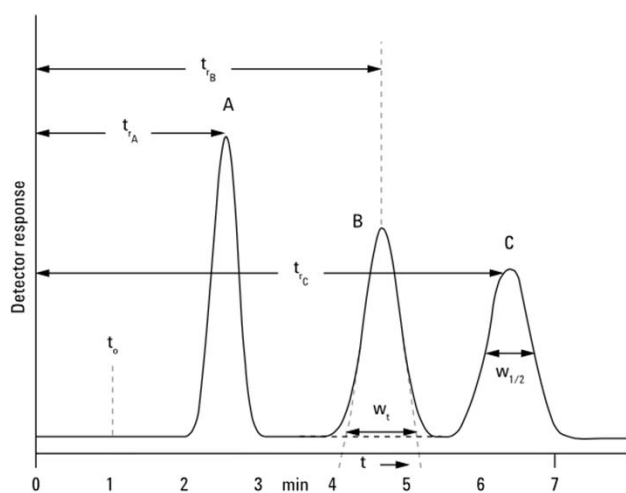
### • Van Deemter

- Height of theoretical plate (smaller the better)
- $HETP = A + B/\mu + C\mu$ , where  $\mu$  is the linear flow velocity
  - Where A is the eddy diffusion term due to non-ideal flow
  - B is diffusion that occurs in the longitudinal direction
  - C is the resistance to equilibration between the stationary and mobile phases

column 50 x 4.6 mm, acetonitrile – water (50:50, v/v),  
analyte toluene



## LC parameters



### Efficiency

$N = 16 (t_R/w_t)^2$   
For Peak B,  $16(4.5 \text{ min.}/0.9 \text{ min.})^2 = 400$  plates  
 $k = (t_R - t_0)/t_0$

### Retention

$k_A = (2.5 - 1)/1 = 1.5$   
 $k_B = (4.6 - 1)/1 = 3.6$   
 $k_C = (6.2 - 1)/1 = 5.2$

### Selectivity (C-B)

$\alpha = k_2/k_1$   
 $\alpha = k_C/k_B = 5.2/3.6 = 1.44$   
 $\alpha = 1.44$

### Selectivity (B-A)

$\alpha = k_2/k_1$   
 $\alpha = k_B/k_A = 3.6/1.5 = 2.4$   
 $\alpha = 2.4$

Agilent handbook of LC

## The pressure equation

$$\Delta P = \frac{\eta F L}{K^0 \pi r^2 d_p^2}$$

Change in pressure  
↓  
 $\Delta P$

Viscosity  
↓  
 $\eta$

Flow rate  
↘  
 $F$

Column length  
↘  
 $L$

Column permeability  
↗  
 $K^0$

Column radius  
↑  
 $r$

Particle diameter  
↘  
 $d_p$

**As the particle diameter is decreased by a factor of two, the backpressure goes up by a factor of four**

Agilent Handbook of LC

## Mobile phases

- **Acidic media**
  - Typically 0.1% formic acid
- **Neutral media**
  - 1-10 mM ammonium acetate or formate
- **Alkaline media**
  - 0.1% ammonium hydroxide (but not with C<sub>4</sub>-C<sub>18</sub> phases)
- **Solvents (water-miscible)**
  - Methanol, acetonitrile, isopropanol (for hydrophobic metabolites)

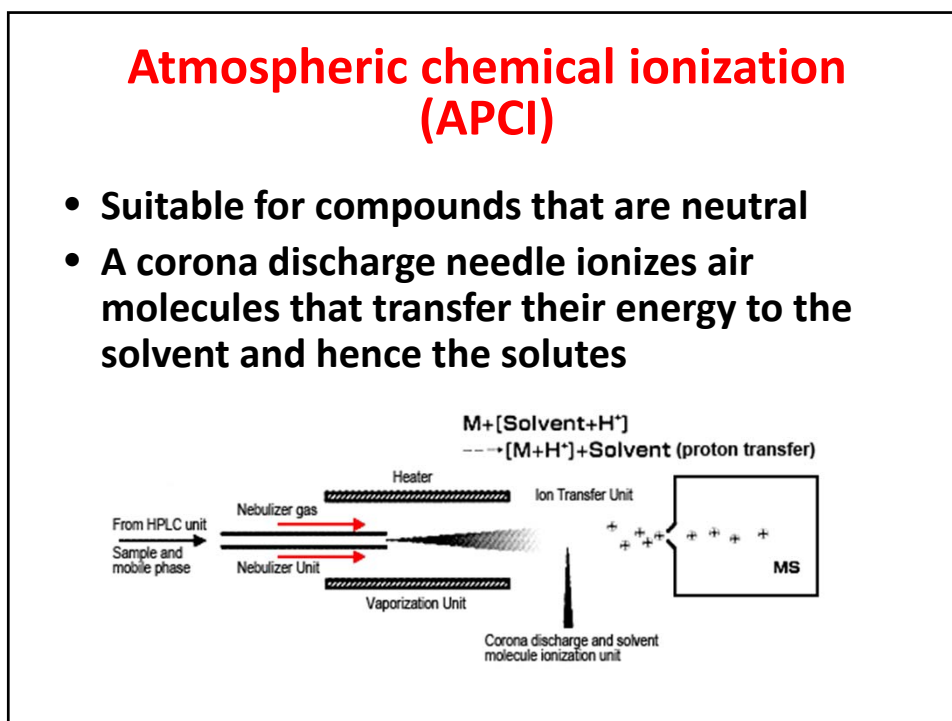
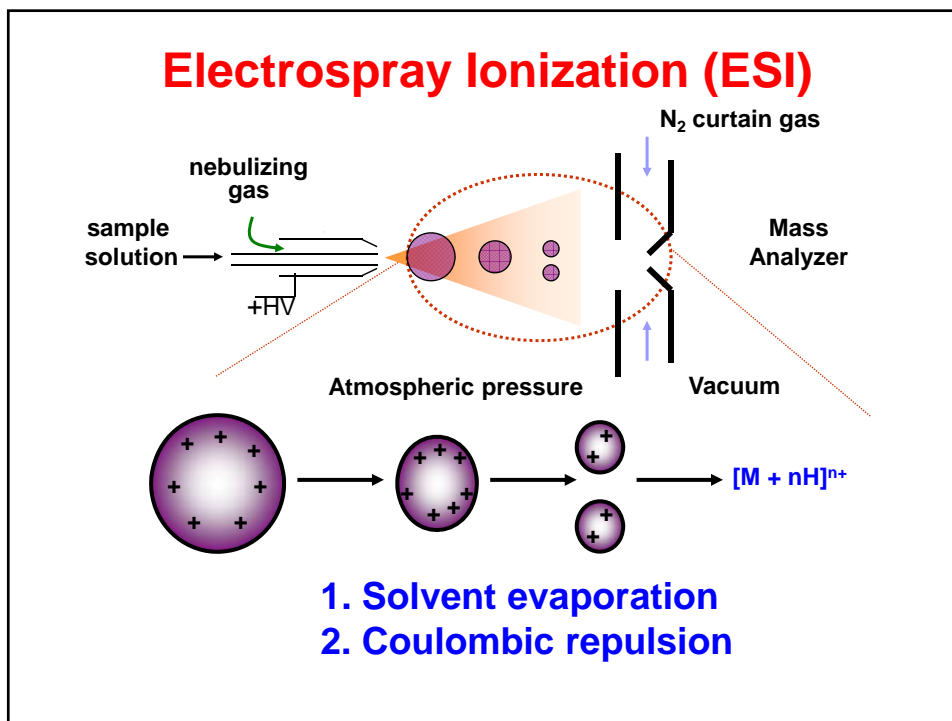
## LC-MS

- The advantage of an effective LC-MS system would be that it would allow thermally unstable compounds, even large ones (such as proteins), to go into the gas phase from liquid solution and into the mass spectrometer
- Importantly, the ionization methods used are soft in nature and molecular ions  $[M+H]^+$  or  $[M-H]^-$  are easily formed (see later re other molecular ions)
- However, there are some compounds that cannot be ionized by LC-MS
  - polycyclic aromatic hydrocarbons, alkanes, waxes.

## LC-MS interface

The key issue is how to transfer ions from the liquid phase into the gas phase while minimizing the transfer of solvent into the mass spectrometer

- For compounds that can be charged, **electrospray ionization (ESI)** is the principal method of choice
- Nebulization of the electrical charged droplets more effectively decreases the size of droplets
  - This allows all aqueous solvents to be processed by the interface
- Heating the spray further increases sensitivity
  - Not used in nanoelectrospray ionization



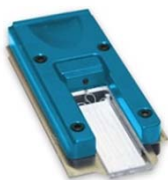
## Guide to LC-MS flow rates

Type	Column ID	Flow rate	Solvent consumed*
Conventional	1.0-4.6 mm	0.050-1.00 ml/min	72-1440 ml
Capillary	0.3-1.0 mm	0.005-0.050 ml/min	7.2-72 ml
Nano	0.05-0.20 mm	100-1000 nl/min	0.144-1.44 ml

Sensitivity in LC-ESI-MS increases in proportion to the inverse of the flow rate. Therefore, there is value in going to lower flow rates – it also saves money on solvents.

## ChipLC versus nanoLC

- A nanoLC column is so thin (75  $\mu\text{m}$  i.d.) it has very little thermal capacity – this leads to variable retention times due to temperature fluctuations in the lab



A column etched in a block of silica can be engineered to have greater physical reproducibility and it has far greater thermal capacity. The CHIP can be placed in temperature-controlled chamber – we operate ours at 45°C – to recover more hydrophobic metabolites

## Engineered microflow LC



Chromatography at flow rates of 5-50  $\mu\text{l}/\text{min}$  using 0.3-0.5 mm ID columns

Very low dead volumes between the sample injection valve and the ESI interface despite the low flow rate

Enables short, reproducible gradients (1-2 min) or up to a 20 min gradient (for metabolomics) at 5  $\mu\text{l}/\text{min}$

## Detector types

Type	Mass range (m/z)	Resolution	Accuracy (ppm)	Time for MSMS (msec)
Quadrupole	20-3000	2,000	50	1000
TOF	unlimited	30,000-40,000	2-3	50 or less
Orbi-trap*	50-6000	80,000-200,000	1-3	200+
FT-ICR*	100-1,500	Up to 1,000,000	<1	1000

\*These detectors depend on ion motion and therefore their performance declines as the acquisition time is shortened. Using a 80 msec MSMS acquisition, mass resolution on an Orbi-trap falls to 17,000. The TOF detector is the preferred one for untargeted analysis. The Orbi-trap and FT-ICR instruments are important for follow-up high mass accuracy experiments.



## Detector combinations

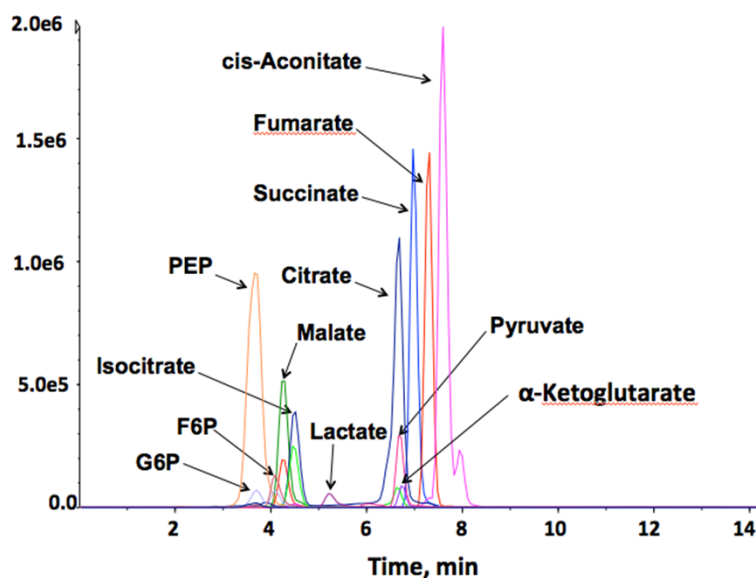
- **Each detector can record a MS spectrum**
  - Not sufficient even with high mass accuracy to identify the metabolite
    - 100s of metabolites can have the same empirical formula (and identical mass)
- **Fragmentation of selected ions creates a MSMS spectrum to distinguish isobaric metabolites**
  - In IDA analysis, molecular ions detected in a quick Hi-Res MS, are “selected” by the quadrupole filter one at a time
  - The ion is fragmented and a MSMS spectrum recorded
    - TOF instruments can record 20 MSMS spectra per second

## Primer for selecting ions for MRM

Peak width (sec)	Cycle time (sec)	Dwell time (msec)	Number of channels
5	0.5	20	25
10	1.0	20	50
5	0.5	10	50
10	1.0	10	100
5	0.5	5	100
10	1.0	5	200
5	0.5	2	250
10	1.0	2	500

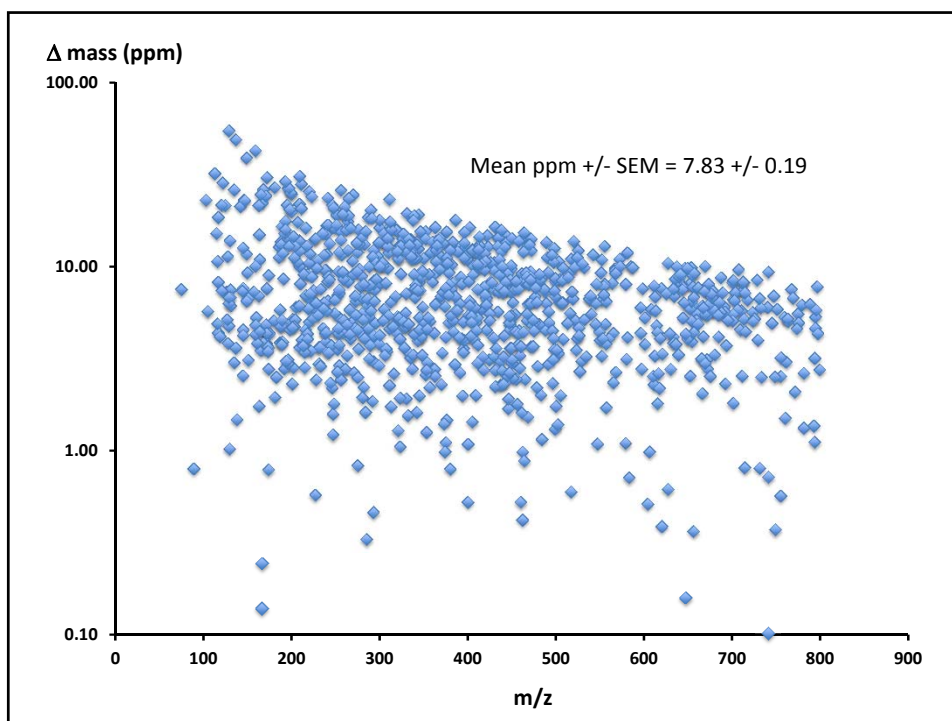
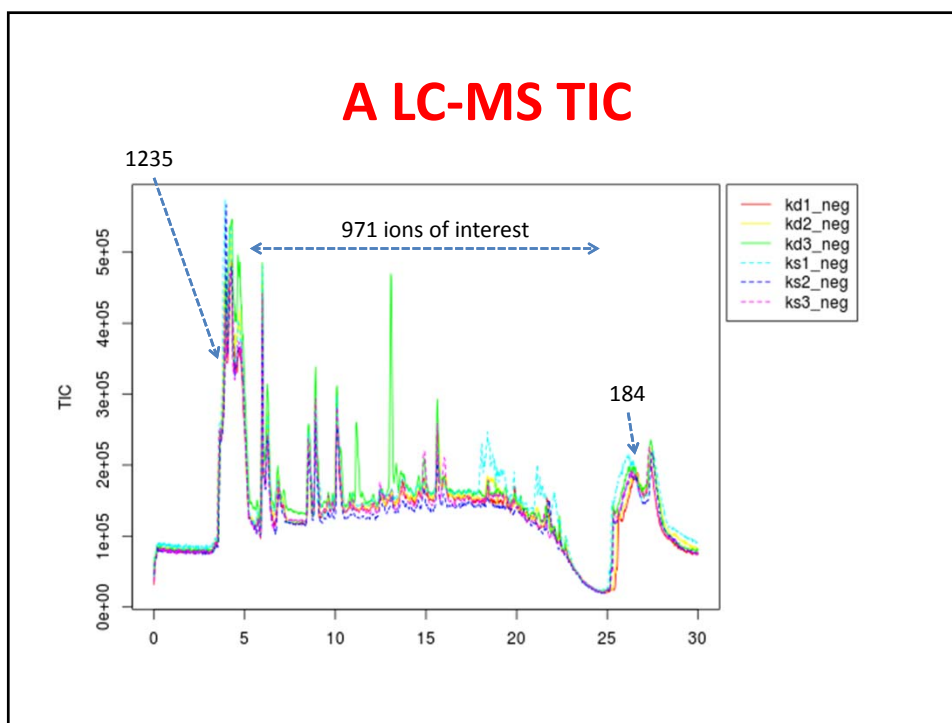
The number of channels can be increased by using timed windows

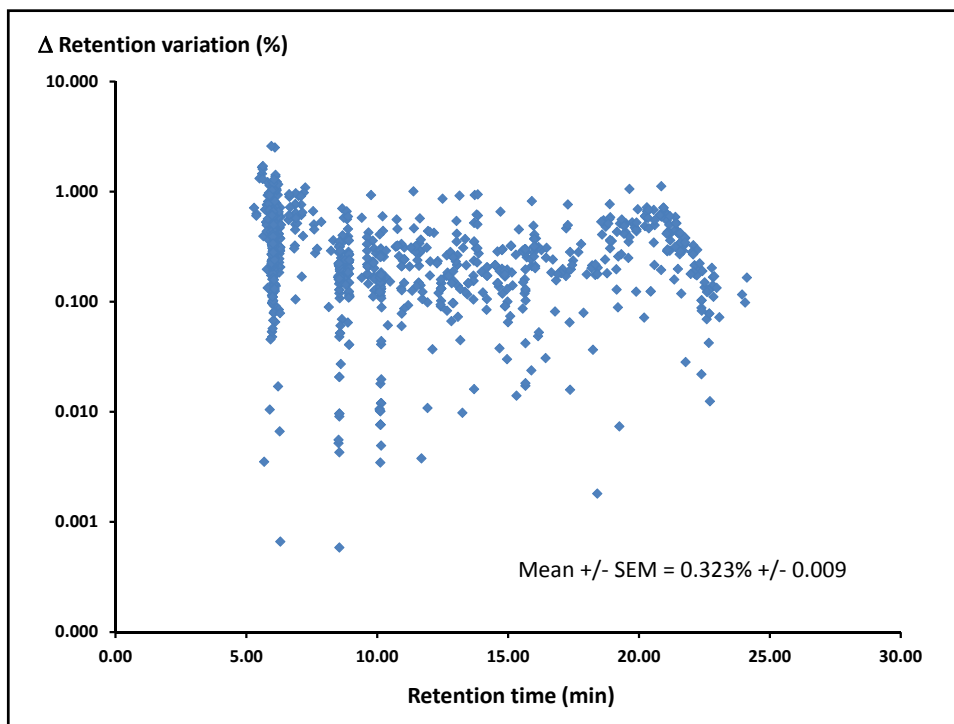
## Combined channels for Krebs cycle




## Untargeted LC-MS

- The instrument of choice is a quadrupole-orthogonal TOF
  - We use an AB Sciex 5600 TripleTOF
- Collection of data
  - Duty cycle of 2 secs (to allow enough data points across a chromatography peak)
  - 0-100 msec high accuracy MS in TOF analyzer (to identify the most intense ions)
  - 100 msec – 2 sec
    - 100 msec MSMS spectra of up to 19 different peaks





## Sampling in metabolomics



**Charles Burant**  
U. Michigan

**Rick Higashi**  
U Kentucky

**Oliver Fienh**  
UC-Davis

**Susan Sumner**  
RTI Intl

**Arthur Edison**  
U. Florida

**Sreekumaran Nair**  
Mayo

**David Wishart,**  
U. Alberta

**Jeremy Nicholson,**  
Imperial College

**The metabolites are  
“the canaries of the genome”**

<http://www.metabolomicsworkbench.org/>

## Metabolism and time

- Not only should metabolites appear in the right place, there is also the question of the importance of the timescale
- Metabolism defects in the heart may be only seconds away from death – rogue waves in metabolism??
- Irreversible damage to the brain may occur in minutes
- Go/No-Go decisions for a cell to divide or apoptose may occur in 10s of mins





**Can we sample fast enough?**

## What about cells in culture dishes?

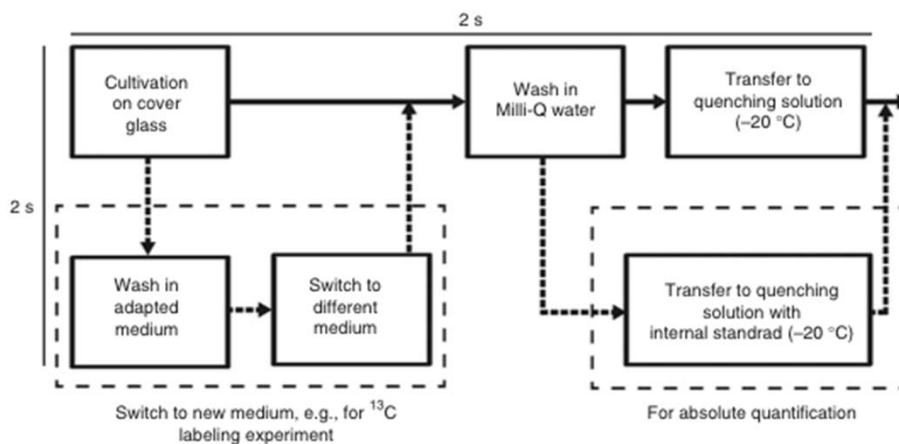
- **Current method we've discussed is to:**
  - Add 10 volumes of ice-cold PBS to the culture dish
  - Swirl and flush
  - Followed by super-cold methanol
- **Is this good enough?**
  - Simple physics says if the "ice-cold buffer" is at 4°C, the addition of the ten volumes will reduce the temperature of the cells to 7.3°C
  - Cool, not cold! (and shaken, not stirred!!)

## Nature Protocols, January, 2015

### Fast sampling method for mammalian cell metabolic analyses using liquid chromatography–mass spectrometry

Giuseppe Martano<sup>1,3</sup>, Nathanaël Delmotte<sup>1,3</sup>, Patrick Kiefer<sup>1</sup>, Philipp Christen<sup>1</sup>, David Kentner<sup>2</sup>, Dirk Bumann<sup>2</sup> & Julia A Vorholt<sup>1</sup>

## Grow cells on cover slips



In this work, about 300,000 cells were grown on each cover slip

Martano et al. Nature Protocols, 2015

## Preparing for culturing

- To prepare cells for the experiment, place an autoclaved cover glass into one of the central wells of a six-well plate and seed the cells on top.
- In our experiments, we seed each well with ~300,000 HeLa cells in 2 ml of DMEM with serum.
- To equally distribute the cells, gently move the six-well plate vertically and horizontally (avoid circular movements, which often result in ring-shaped cell layers).
- For absolute quantification, it is necessary to prepare additional cell samples for cell counting.

Martano et al. Nature Protocols, 2015



## Culturing conditions

- Place the six-well plates in the incubator, and let the cells rest to attach and form a monolayer.
- Before the experiment, it is advisable to exchange the medium for fresh medium to ensure defined medium conditions (**changing to medium without serum has the advantage of bringing cell division to a stop and synchronizing the cells**).
- In our experiments, we seeded cells 22 h before the experiment and exchange the medium for DMEM without serum at 16 h and again at 3.5 h before the experiment.

Martano et al. Nature Protocols, 2015

## Step-by-step

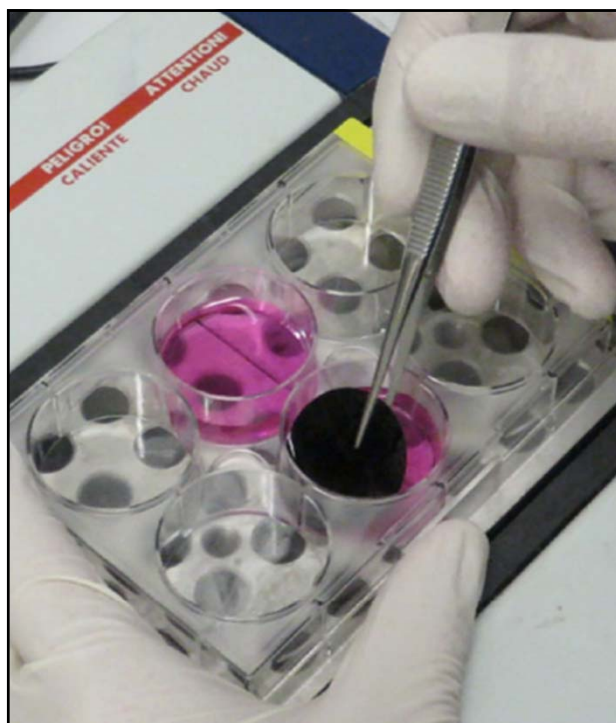
- The initial cell density we typically use is  $2 \times 10^8$  cells per liter, and 2 ml is required per analytical sample.
- It would be possible to use fewer cells without adverse effects, assuming that the sensitivity of your LC-MS system is similar to that used in our laboratory.

Martano et al. Nature Protocols, 2015

## Washing the cells

- Ultrapure water is used to wash HeLa cells on the cover glass before quenching and metabolite extraction.
- For further manipulations of the cells (e.g., dynamic labeling experiment), adapt the washing solution accordingly (e.g., DMEM without glucose).

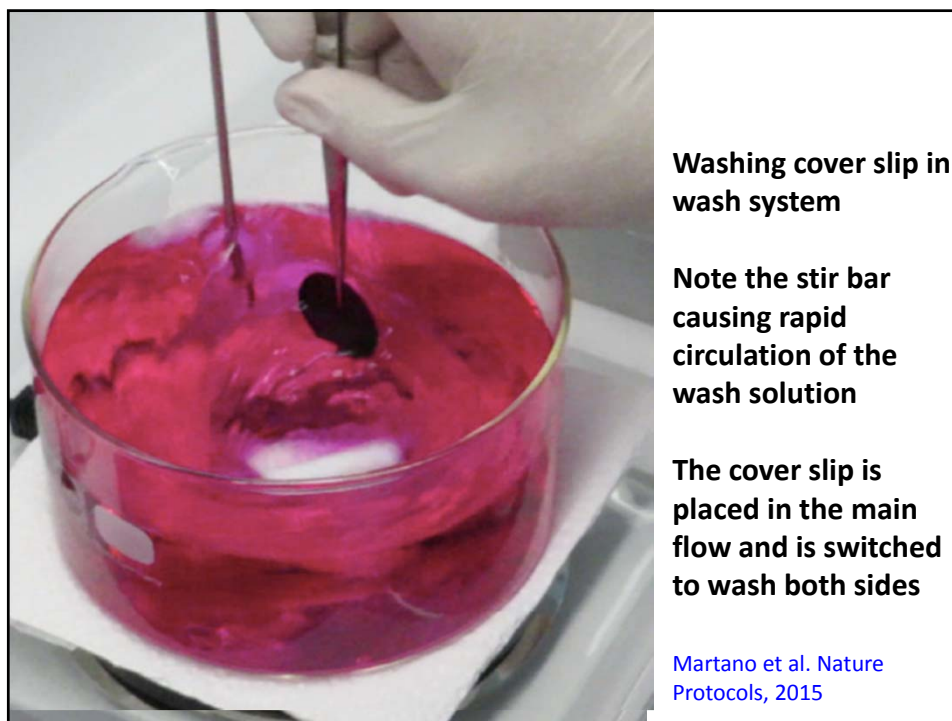
Martano et al. Nature Protocols, 2015



Cells on cover slip in  
6-well plate

Handle with  
tweezers

Martano et al. Nature Protocols,  
2015



## Trouble shooting

- ***Frequent loss or breakage of the cover glass during the washing step:*** ensure that you do not accidentally touch the rotating stir bar with the cover glass.
- **To prevent the strong current from sweeping away the cover glass, do not hold it vertical to the current when you are tilting the cover glass.**
- **You may have to reduce the speed of the magnetic stirrer or use a shorter magnetic stir bar to slow down the current.**

[Martano et al. Nature Protocols, 2015](#)

## Quenching solution

- Mix acetonitrile, methanol and 0.5 M formic acid at a ratio of 2:2:1 (vol/vol).
- The solution may be stored at room temperature (20°C) for several weeks when it is tightly sealed.
- The amount required for the sampling of one cover glass is 8 ml.

Martano et al. Nature Protocols, 2015

## Extraction method

- Eight ml of the quenching solution is pipetted to one well of a six-well plate, and it is cooled to -20 °C in a freezer.
- For better temperature buffering, 2 ml of water is pipetted into each of the five other wells, and 4 ml is pipetted into the inter-well space of the plate.
- Several samples in the same six-well plate are not advisable, as samples are processed one after the other and incubation conditions are difficult to maintain outside the incubation chamber.

Martano et al. Nature Protocols, 2015

## Important steps - quenching

- Drop the washed cover glass into the quenching solution with the cells facing upward.
- Cover glasses must be handled with care to avoid breakage when lifting them with the pointed tweezers (glass often adheres strongly to the well bottom) and to avoid losing the cover glass during the washing in water (or medium).
- Practice is needed to achieve thorough and quick (2 s) washing, which is why these steps should be practiced before performing the real experiment.
- Because each washing step slightly contaminates the wash water (calculate 0.2–0.5 ml of residual medium per cover glass), we recommend replacing the wash water after every second or third sample (and immediately in the case a cover glass is accidentally dropped into the beaker).

Martano et al. Nature Protocols, 2015

## Extraction

- Use a cell scraper to scrape off the cells from the cover glass. Pipette the suspension, with as little left over as possible, into a 50-ml centrifugation tube.
- Sonicate the sample using a sonication bath filled with ice water, and subsequently incubate the cells for 15 min on ice for metabolite extraction.
- Freeze the sample using liquid nitrogen and keep it in liquid nitrogen until ready to freeze dry.
- Freeze-dry the samples until complete dryness is obtained.

Martano et al. Nature Protocols, 2015

## Freeze-drying issue

- Depending on the extraction solution, the quantity of organic solvents—and in particular the quantity of methanol with its very low freezing point—can cause partial thawing of the sample, and splashing could occur during freeze drying.
- It is therefore important to store the samples in liquid nitrogen at least 30 min before freeze drying.
- Sample loss resulting from splashing is avoided by using 50-ml conical centrifugation tubes, as recommended in this protocol.

Martano et al. Nature Protocols, 2015

## Freeze drying problems

- *Loss of sample after freeze drying:*
  - The samples were not cold enough when placed in the freeze dryer, and/or the openings of the 50-ml centrifugation tubes were too big.
  - In the latter case, we recommend piercing the plastic cap of the tube, e.g., with a disposable needle.
- Remember to replace the caps with new caps after freeze drying.

Martano et al. Nature Protocols, 2015

## Getting ready for HPLC (1)

- **Add ice-cold ultrapure water to the dried sample to obtain a final biomass concentration of ~2,000 cells per  $\mu\text{l}$** 
  - for HeLa cells, we typically obtain ~600,000 cells per cover glass, corresponding to a volume of 300  $\mu\text{l}$  per sample to be added.
- **Press the sample onto a vortex mixer and shake the tightly closed tube until the sample is completely resuspended.**

Martano et al. Nature Protocols, 2015

## Getting ready for HPLC (2)

- **Spin down the sample to the tube cone, and transfer the complete sample into a 1.5-ml conical centrifugation tube. Centrifuge it for 10 min at 20,400g and 4 °C.**
- **Transfer the sample supernatant into an empty 1.5-ml conical tube.**
- **Before analysis, dilute the sample 1:4 with HPLC solvent A. When using 200- $\mu\text{l}$  microinserts for the HPLC vials, add a 10- $\mu\text{l}$  sample aliquot to 30  $\mu\text{l}$  of solvent A.**

Martano et al. Nature Protocols, 2015

## LC-MS points (1)

- Analyze the samples by LC-MS. Inject 1  $\mu\text{l}$  of the sample using a pre-programmed instrument method comprising the LC gradient and MS parameters.
  - We recommend full-loop injections with a 1- $\mu\text{l}$  sample loop if the sample amount is not limited.
- When performing full MS scans with an FTMS instrument, all mass traces of the complete scan range are measured.
- Because no specific scan events are predefined, the same LC-MS method can be applied for label-free approaches (e.g., metabolite identification), absolute quantification based on isotope ratio analysis, and dynamic labeling experiments.

Martano et al. Nature Protocols, 2015

## LC-MS points (2)

- Ensure that MS data acquisition speed is sufficient for accurate peak area quantification (at least ten data points per peak) when applying other LC-MS methods.
  - This is of major importance when applying ultraperformance LC, which results in narrow peak width down to 1 s (full width at half maximum) requiring an acquisition frequency of at least 10 Hz.
- Acquisition frequency can be crucial for FTMS instruments in which mass resolution is proportional to dwell time in the analyzer (LTQ-Orbitrap classic 1-s dwell time at  $R = 60,000$ ) but also for QqQ instruments in which many mass transitions per cycle time are measured (e.g., 50 transitions measured with 25-ms acquisition time per mass transition).

Martano et al. Nature Protocols, 2015



## nanoLC-MS method

- Ion pair reversed-phase chromatography is performed on a nano-2D ultra LC system (Eksigent Technologies) using a 0.1-mm ID nano-LC column. We recommend shortening the column to 10.5 cm using an appropriate capillary cutter. Separation is performed at room temperature with a solvent flow rate of 0.5  $\mu\text{l}/\text{min}$ .
- The initial mobile phase composition is 97% solvent A and 3% solvent B (see Reagent Setup), and a linear gradient is applied after sample injection with eluent B varying as follows: 3% at 0 min, 90% at 25 min, 90% at 30 min and 3% from 31 to 41 min (reconditioning).
- The autosampler temperature is set to 4 °C. For full-loop injection, the injection valve is equipped with a 1- $\mu\text{l}$  loop. For full-loop injection, a vial containing 1 ml of solvent A is placed in one Eksigent autosampler vial tray predefined 'reagent position,' e.g., position A1 is required. The sample (5  $\mu\text{l}$ ), followed by 1.5  $\mu\text{l}$  of the reagent, is loaded to ensure that the sample loop is completely filled with the sample. Subsequently, the sample is injected for 2 min at 0.75  $\mu\text{l}/\text{min}$ . Subsequently, the linear gradient is started.

Martano et al. Nature Protocols, 2015

## Mass spectrometer

- LTQ-Orbitrap classic instrument equipped with nanospray housing and dynamic nanospray probe NSI-1 (Thermo Fisher Scientific). For nanospray ionization, the column is connected via liquid junction to a silica tip emitter.
- Samples are measured in the negative FTMS mode at  $-1.9$  kV.
  - Further source settings are as follows: capillary temperature, 150 °C; tube lens,  $-100$  V; and capillary voltage,  $-10$  V. FTMS full maximum injection time is set to 500,000 and automatic gain control (AGC) full-scan injection waveform is enabled.
- Acquisition is performed in FTMS scan mode by applying a scan range of 150  $m/z$  to 900  $m/z$  at a standard resolution,  $R = 60,000$ , which covers most of the core metabolites.
  - Note the rule for efficient ion transfer into the Orbitrap mass analyzer (for this instrument,  $\min(m/z)/\max(m/z) < 1/6$ ).

Martano et al. Nature Protocols, 2015

## LC-MS problems (1)

- ***Frequent clogging of the column:*** the centrifugation step was not sufficient to completely remove the cell debris. Centrifuge the sample supernatants again (20,400g, 10 min at 4 °C) before diluting them with solvent A (see Step 16)
- ***Low spray stability after mounting the new column during sample analysis:*** there could be air in the column. Run blanks until the spray is stable over the complete runtime.
- ***Low spray stability occurs during a series of analyses:*** replace the tip emitter.

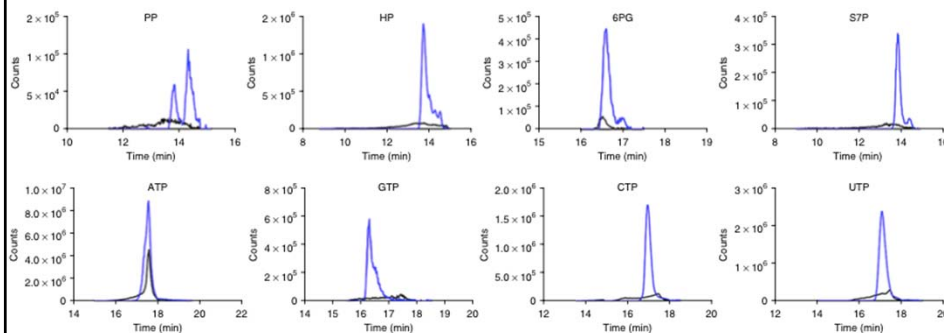
Martano et al. Nature Protocols, 2015

## LC-MS problems (2)

- ***Low peak resolution and significant retention time shifts:*** biomass equivalent in sample is probably too high.
  - We recommend spiking defined amounts of metabolite analogs (e.g., halogenated nucleotides) or metabolites that are not formed by the investigated organism into the sample before analysis.
  - Such standards allow the characterization of sample matrix effects by comparing their signal responses in the sample matrix and those of pure standards in solvent A.
- ***Low signal intensity:*** injected biomass amount could be too low, or the washing step was not performed properly.

Martano et al. Nature Protocols, 2015

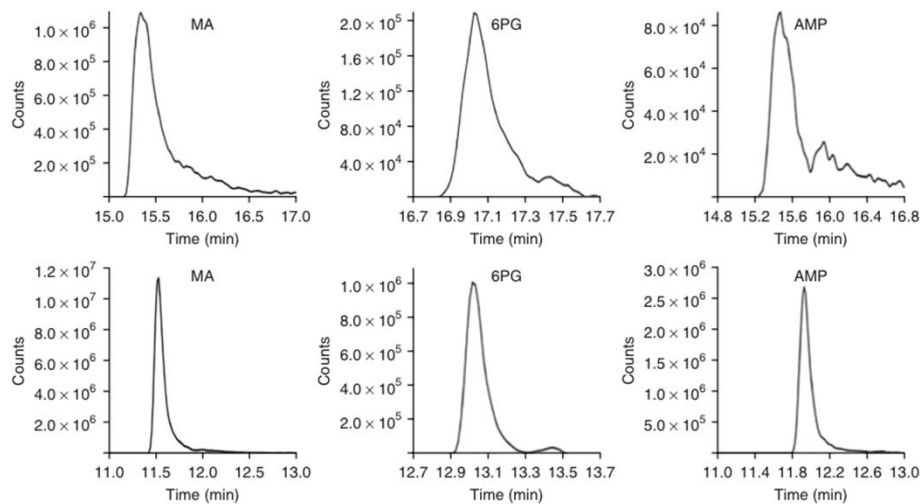
## LC-MS metabolites



Each chromatogram has a black (unwashed cells) and blue (washed cells) trace. Washing is critical.

Martano et al. Nature Protocols, 2015

## Going from 5 $\mu\text{m}$ to 2.7 $\mu\text{m}^*$ particles



\*Core shell particles

Martano et al. Nature Protocols, 2015

Metabolite	Metabolite concentration (fmol per cell)	
	Unwashed samples	Washed samples
ATP	29.90 ± 2.18	25.01 ± 1.55
ADP	2.69 ± 0.19	2.72 ± 0.24
AMP	0.43 ± 0.07	0.36 ± 0.05
Hexose-6-phosphate	0.68 ± 0.06	0.59 ± 0.02
Pentose-5-phosphate	0.08 ± 0.02	0.05 ± 0.01
Phosphoenolpyruvate	0.04 ± 0.02	0.05 ± 0.01
Fructose-1,6-bisphosphate	1.01 ± 0.10	0.77 ± 0.05
6-Phosphogluconate	0.34 ± 0.01	0.41 ± 0.02

Concentrations were determined using the isotope ratio-based approach. Data are presented as the mean ± s.d. of five biological replicates. [Martano et al. Nature Protocols, 2015](#)