Untargeted and Targeted Metabolomic Workflows Bioanalytical Tools for Clinical Research

Designed for non-LC MS experts – What problems can we use this for?

General Metabolomics Workflow – Agilent Technologies Centric
Delivered February 2024

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Applied Omics & Life Sciences LLC
Agilent Technologies Applications Contractor

Multi-OMICS Research – Focus on Metabolomics

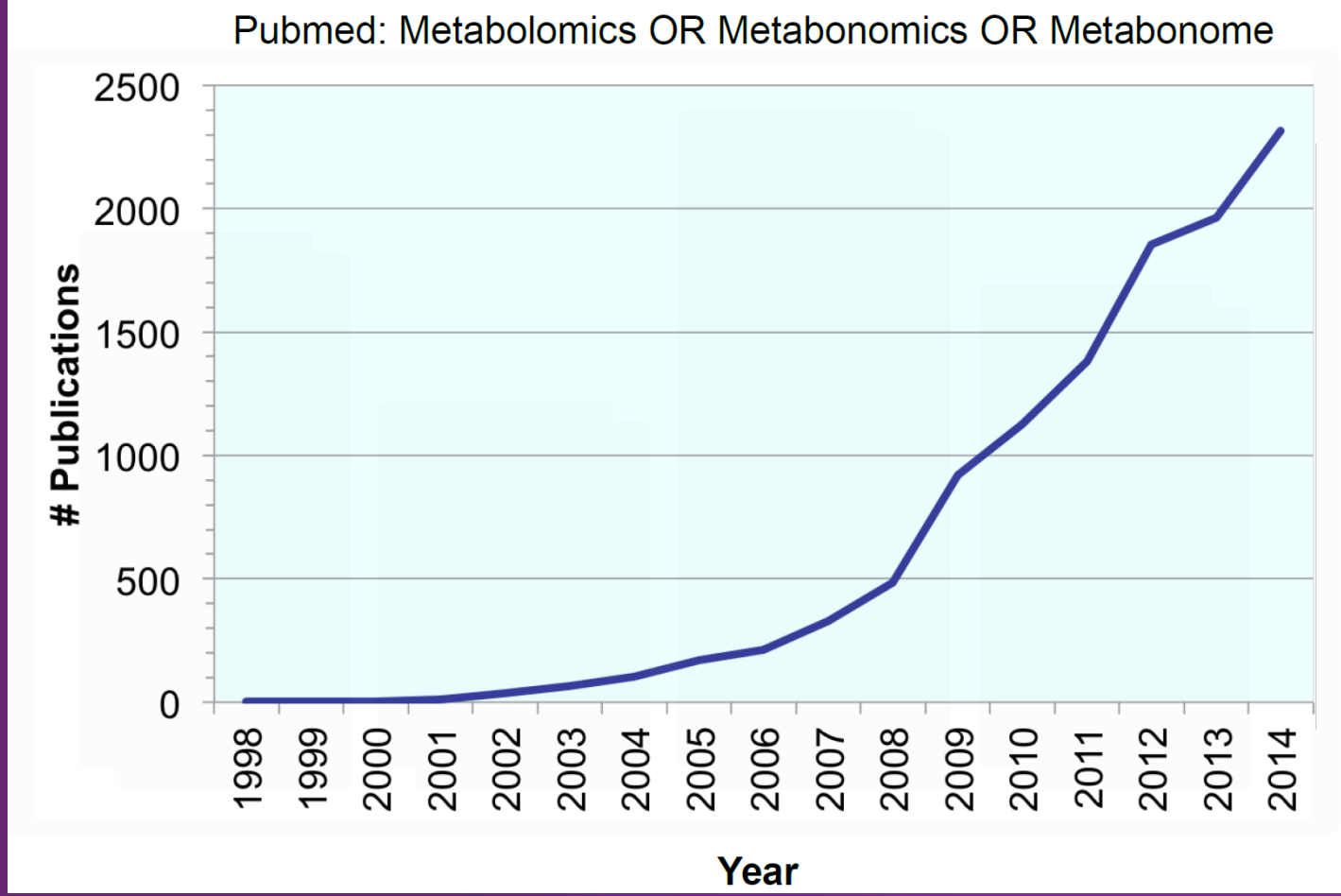
Presentation Outline

I. Metabolomics Workflow Overview
II. Hypothesis / Question(s) and Pathway

(Workflow Steps and Experimental Design)

III. Types of Samples and Extraction (A)
IV. “Feedback” Loops with Results for Decisions
V. Metabolome Chemical Diversity and Chromatography (B)
VI. Why the 6546 QTOF LC MS system (C)
VII. Bioanalytical Approach to Oncology Systems
VIII. Example Results and Oncology Applications

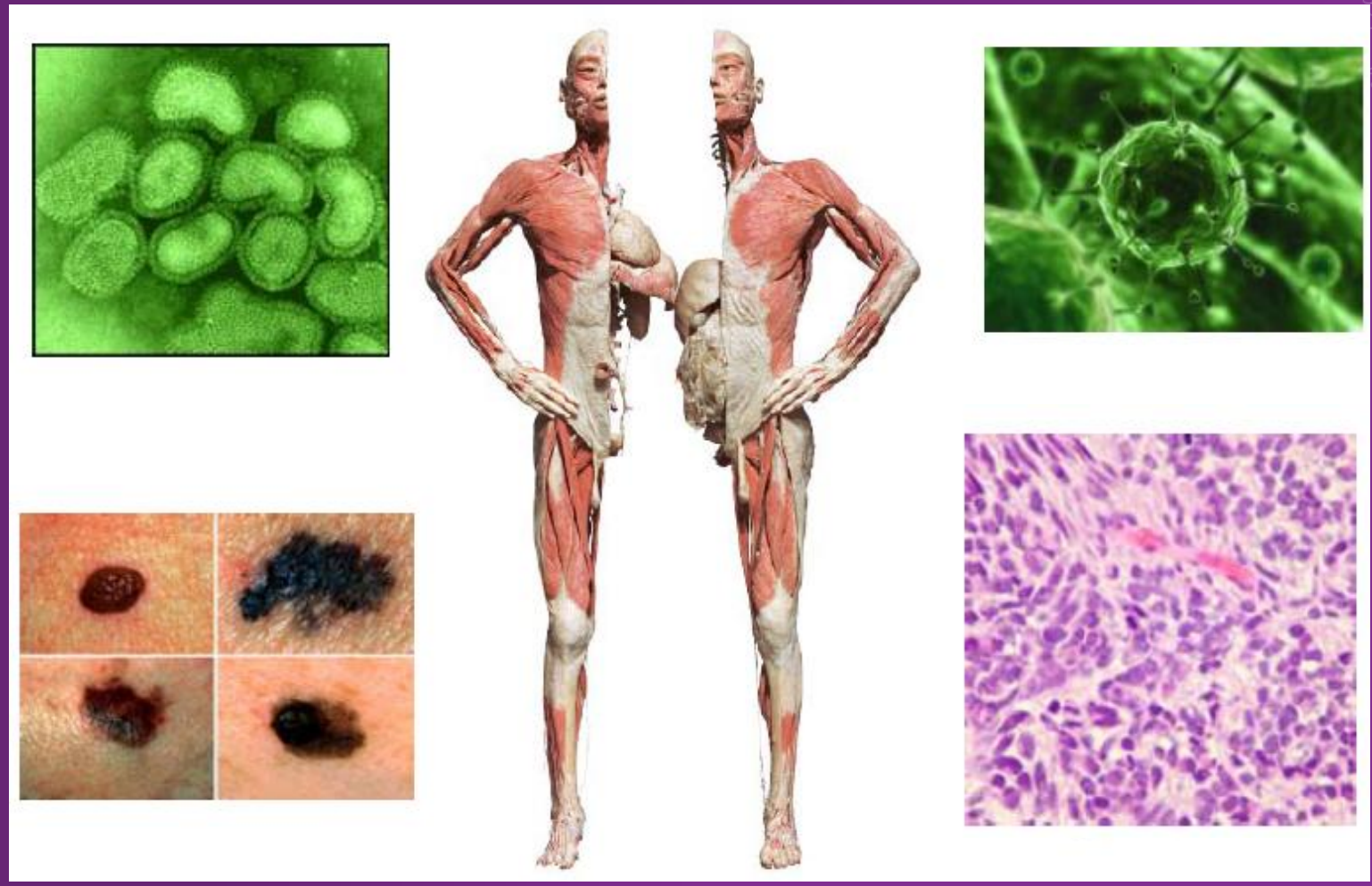
Metabolomics Is Growing



Google Scholar Search by Year Metabolomics

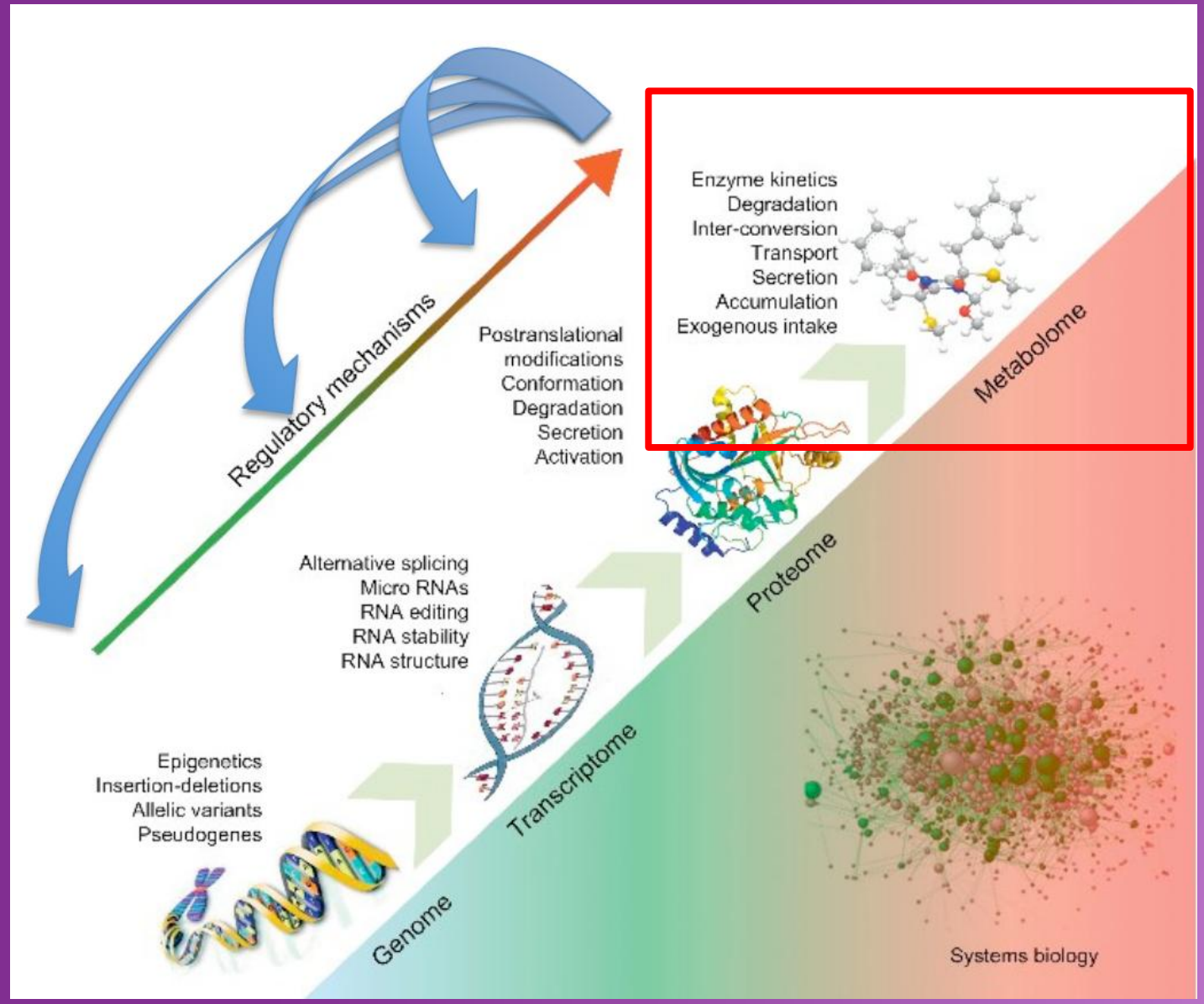
2014 – ~20,000
2018 – ~43,700
2020 – ~59,000
2023 – ~83,600

Omics Research and Life Sciences – *The Questions We Ask*



For Life Sciences Research Only, Not for Diagnostic Purposes

Multi-OMICS Research – Focus on Metabolomics



Metabolomics

BIOCHEMICAL VARIATION INTEGRATES GENES AND ENVIRONMENT

GENOMICS

Our genes can suggest what diseases we *might* be predisposed to, but it's an incomplete picture of human health.

METABOLOME

The complete status of an individual that can be used to assess health and disease.

LIFESTYLE/ENVIRONMENT

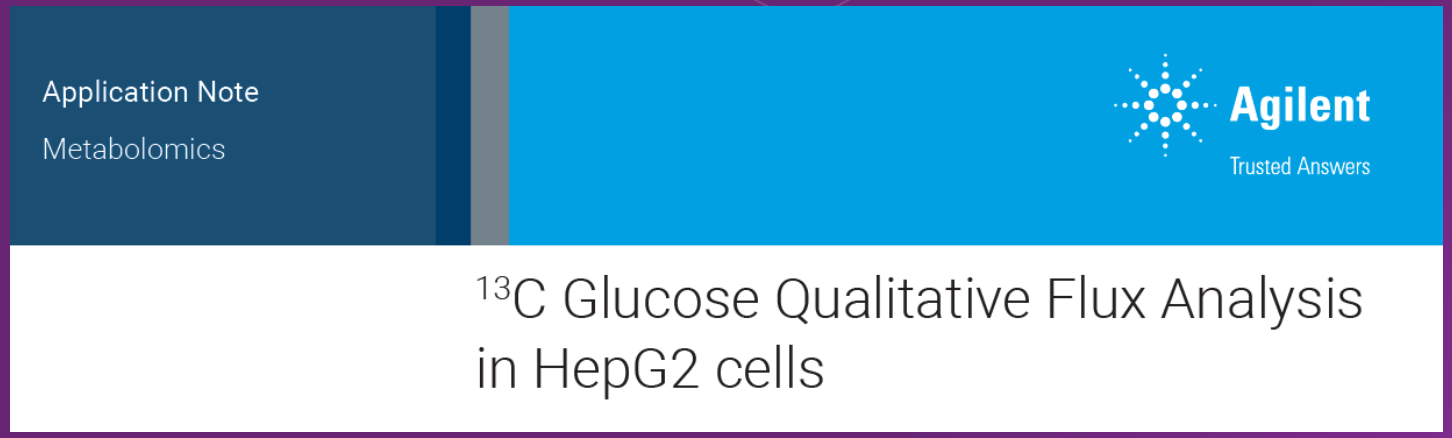
External factors like diet, exercise, medications, microbiota and even where we live influence the state of our metabolome.

Application Teaser

Pivot from the workflow for a moment

Sum = composition

Sum = concentration



Example Application

- Data Collected with a 6546 LC/Q-TOF
- Flux Analysis in human carcinoma cell lines with U-13C glucose tracer.
- Effect of Pyruvate carboxylase knockdown on glucose flux in TCA cycle in HepG2 cells.

Think in terms of Bioanalysis

- Philosophy - Think in terms of:
- ***sample type***
- ***pathways of interest***
- ***biomolecular pathways***
- ***related to type of oncology research***

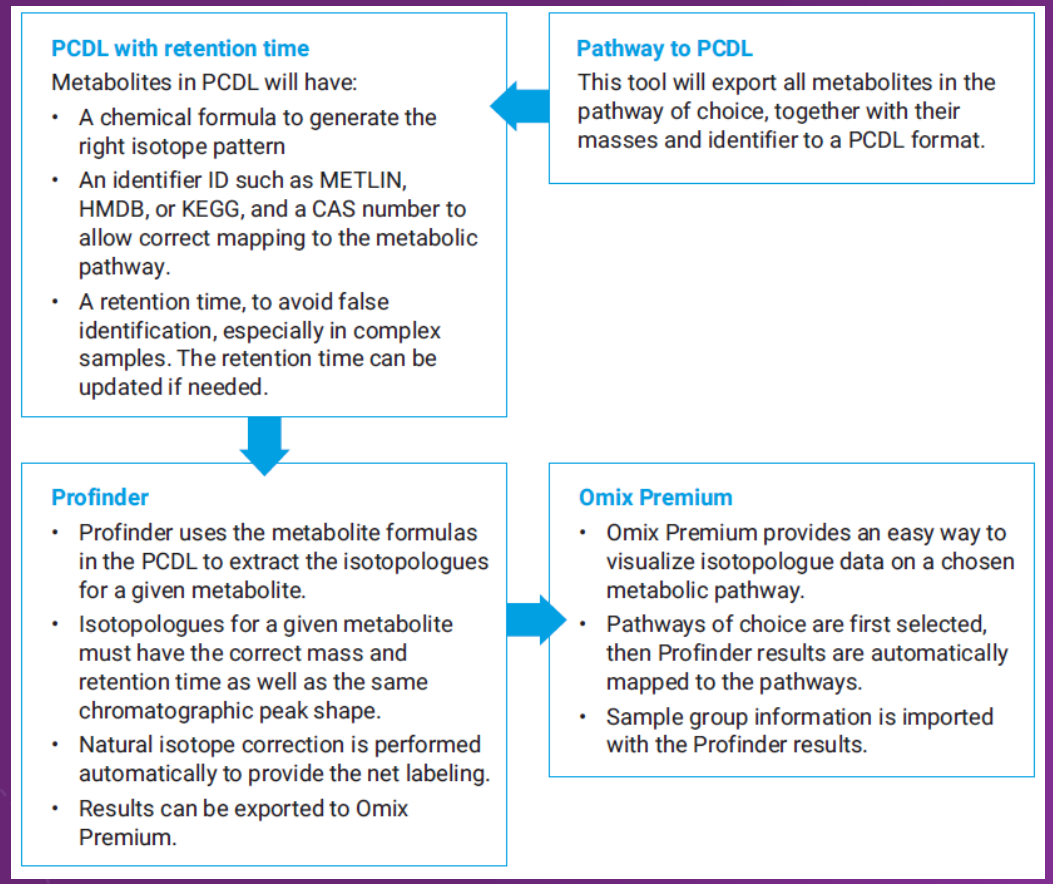
Application Note

Metabolomics

Agilent

Trusted Answers

^{13}C Glucose Qualitative Flux Analysis in HepG2 cells



Software for Data Extraction and Qualitative/Quantitative Analysis

Think in terms of Bioanalysis

- Philosophy - Think in terms of:

- *sample type*
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One of several streamlined workflows for data extraction and analysis
→ Flux and metabolism studies

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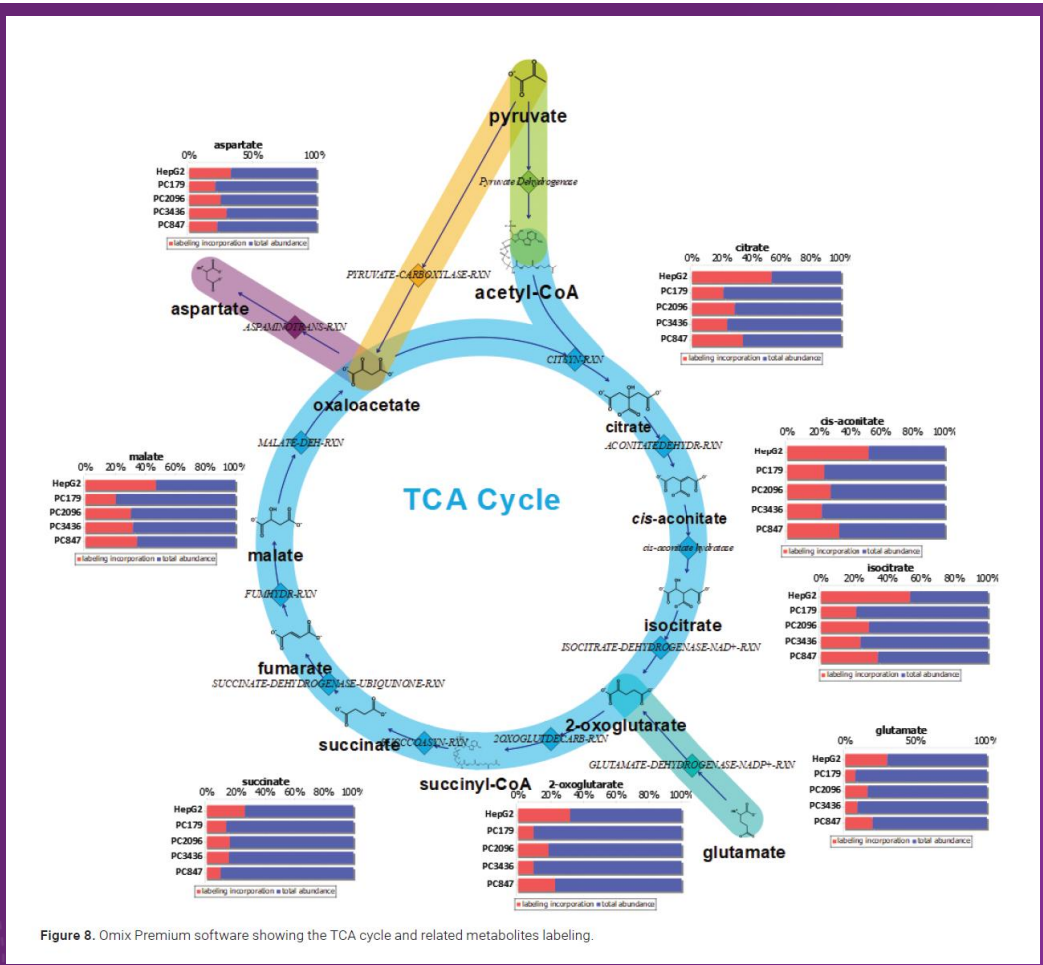

Figure 8. Omix Premium software showing the TCA cycle and related metabolites labeling.

Software for Data Extraction and Qualitative/Quantitative Analysis

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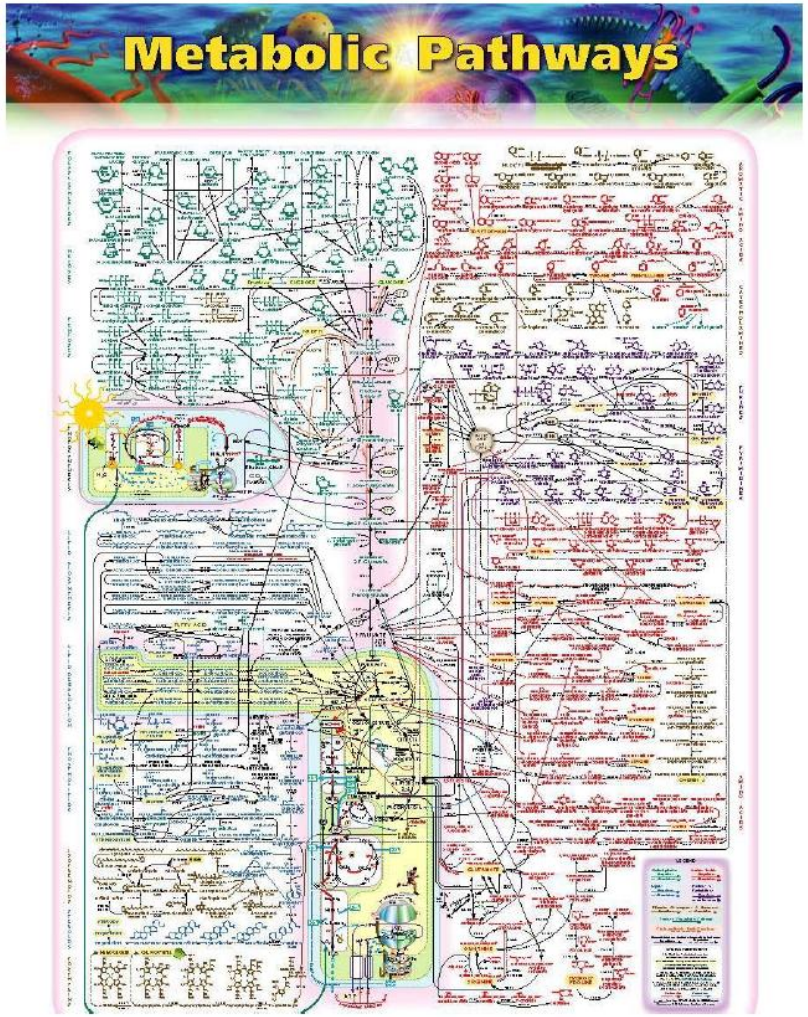
One of several streamlined workflows for data extraction and analysis
→ Flux and metabolism studies

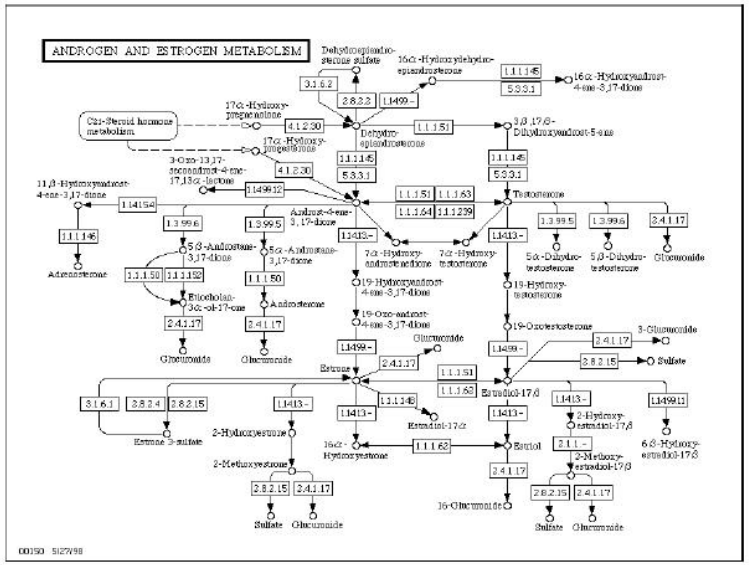
What is your pathway of choice?

See this as finite amount of possibilities in chemical space.

Change in molecule metabolism (errors) for oncology seen in pathway. often more than one molecule



https://webspace.utexas.edu/yg387/interaction.htm



http://kcampbell.bio.umb.edu/lecturel.htm

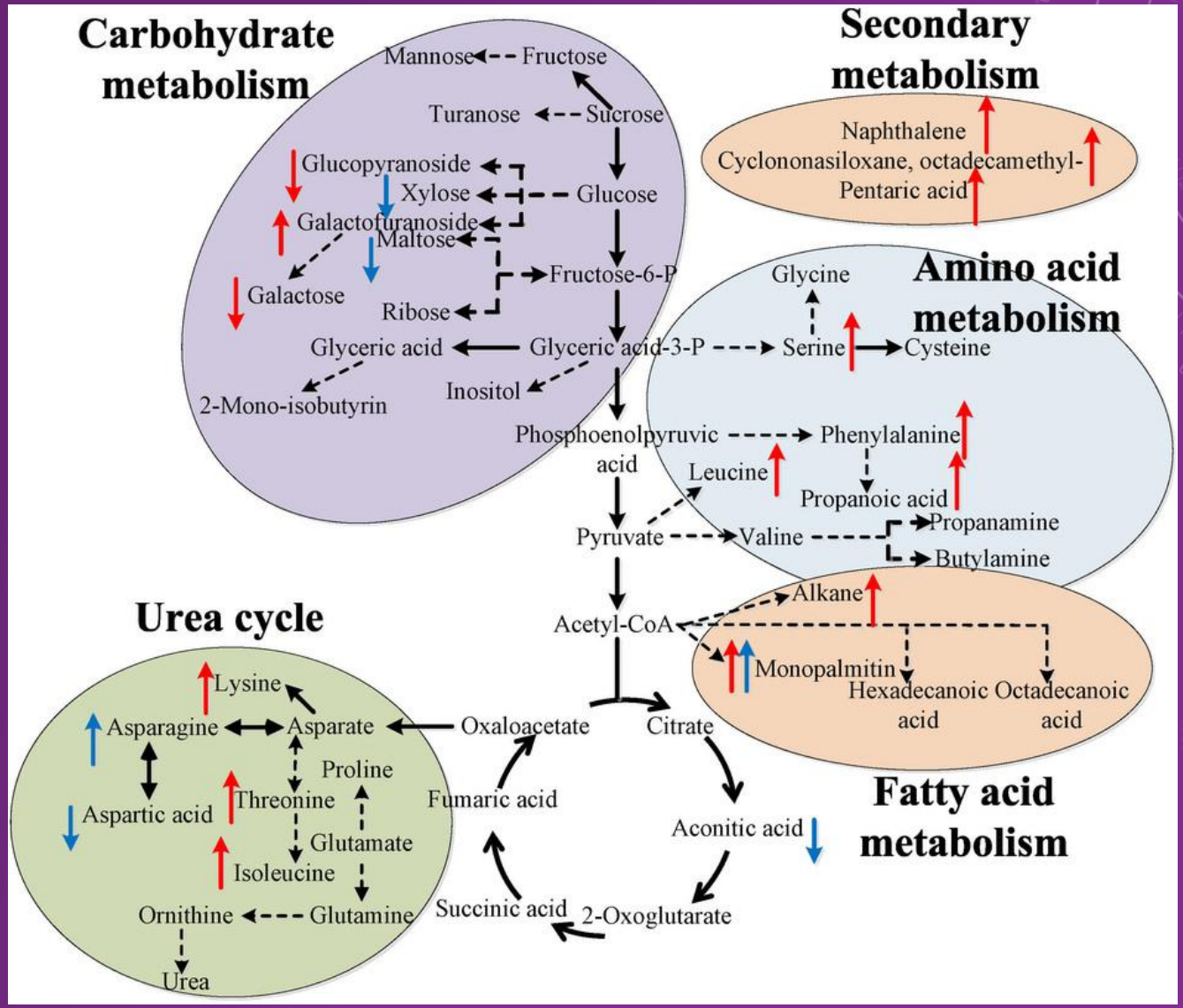
What is your pathway of choice?

See this as finite amount of possibilities in chemical space

Only so many ways that we can put molecules together

Endogenous vs Exogenous Molecules

 # 1 difficulty in Clinical Research for small molecules is the Exogenous (Life Style) inputs from patients in cohorts



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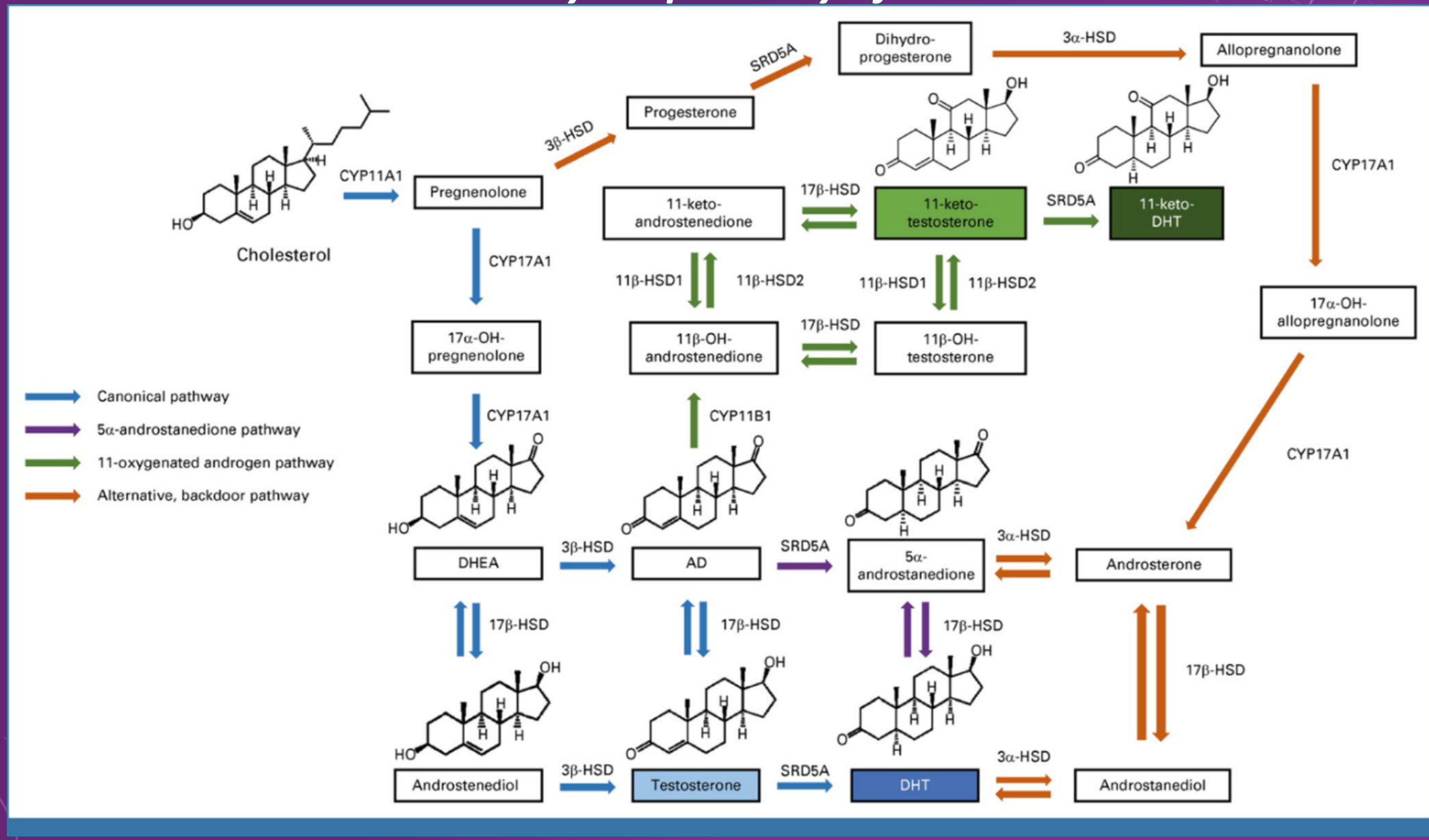
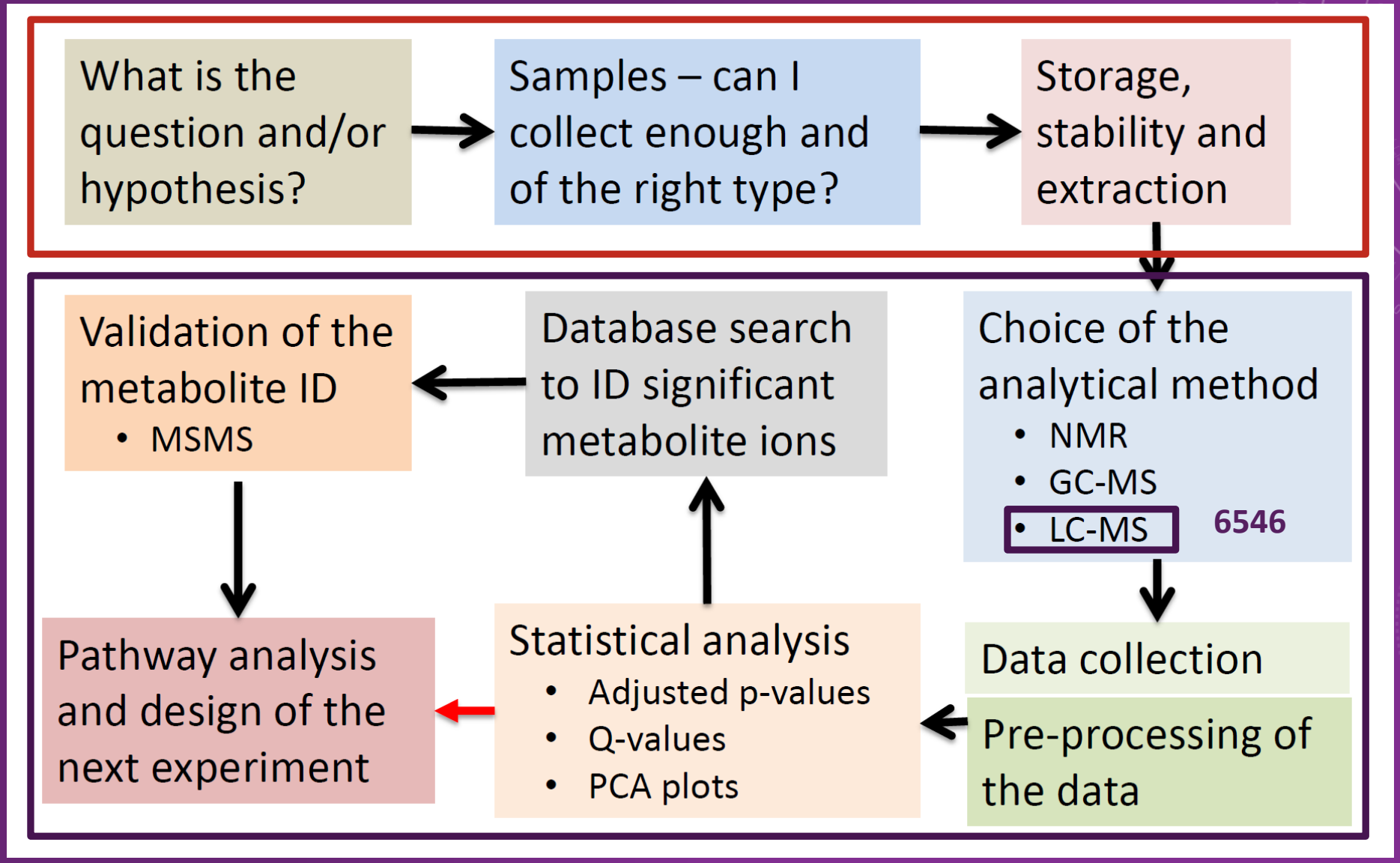


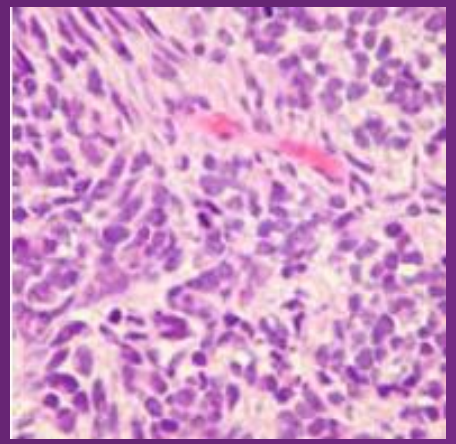
Samples and Preparation

Omics Research and Life Sciences – *The Questions We Ask*



Sample Type

- Blood Plasma/Serum
- Organ Tissue
- Siliva
- Urine
- etc.



Sample Types and Matrix Effects

Largest Variance (When Standard Methods Used) is due to possible "Matrix Effects"

i.e. Blood Mouse vs Human
i.e. Blood from different cancer type
i.e. Tissue vs Cell Culture Model
i.e. Tissue vs Blood
i.e, Tissue A vs Tissue B

Part of development and a feedback loop early during experiment development

Research to Translational *to Diagnostic* → Plan – Develop – Implement – *(Validate?)*

The Power to Design your own Workflow for your system
→ or adopt previous standard workflows from the community.

What is the end goal of your metabolomics experiment – *avoid fishing expeditions*.
But don't be scared to design and optimize. (*It's called research not search*)

Sample Type Preparation Protocols: *20+ years of protocol development*

Plasma/serum

- 100 µL of plasma/serum
- 20 µL of IS solution
  - 40 µg/mL Trp-D3
  - 4 µg/mL Leu-D10, Creatine-D3, Caffeine-D3 and Salicyclic acid-D4
- 800 µL of 8:1:1 ACN:MeOH:Acetone)
  - Chill for 30 min in fridge
- Mix
- Centrifuge 20,000 rcf, 10 min, <10C
- Transfer 750 µL of supernatant
- Dry with gentle stream of N_2
- Reconstitute in 100 µL of 0.1% FA in water (Mobile phase A)

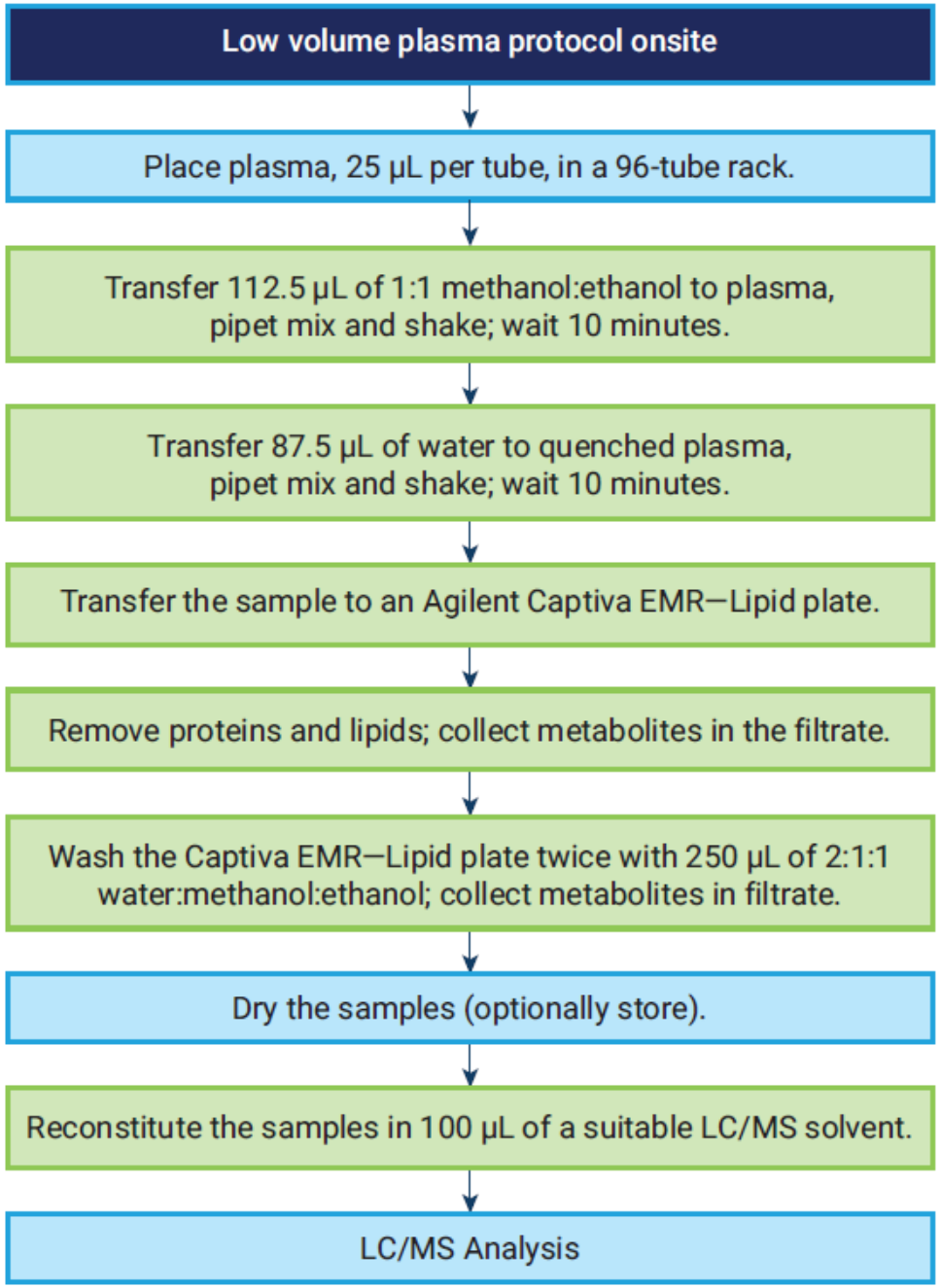
Brain tissue

- 1-10 mg of brain tissue
- 500 µL of chilled 50/50 MeOH/water w/10mM Amm Acetate
- 20 µL of IS solution
- Homogenize
- Centrifuge, transfer 450 µL of supernatant
- Dry and reconstitute in 50 µL (Mobile phase A)

Plant tissue (stool is similar

- 30 mg of plant tissue
- 20 µL of IS mix
- 750 µL of Meoh/750 µL of 10mM Amm Acetate
- Vortex, sonicate
- Centrifuge 10 min, 20,000 rcf
- Transfer 1 mL of supernatant to LC vial

ocols: *20+ years of protocol development*



Chromatographic conditions and MS parameters

Table 1. Agilent 1260 Infinity II Prime LC conditions.

Parameter	Value
Analytical Column	Agilent InfinityLab Poroshell 120 HILIC-Z, 2.1 mm × 150 mm, 2.7 µm, PEEK-lined (p/n 673775 924)
Column Temperature	25 °C
Injection Volume	10 µL
Autosampler Temperature	4 °C
Needle Wash	6 seconds in wash port (50:50 water/methanol)
Mobile Phase	A) 10 mM ammonium acetate in water with 2.5 µM Agilent InfinityLab deactivator additive, pH = 9 B) 10 mM ammonium acetate in water/acetonitrile 15:85 (v:v) with 2.5 µM InfinityLab deactivator additive, pH = 9
Flow Rate	0.25 mL/min
Gradient Program	Time %B 0.00 96 1.00 96 4.50 88 7.50 88 8.00 86 13.00 86 16.00 82 22.00 65 23.00 65 23.50 96 25.00 96
Stop Time	25.00 min
Post Time	3.00 min
Observed Column Pressure	170 to 330 bar

Sample Type Preparation Protocols - *20+ years of protocol development*

Agilent Specific Workflows for Metabolomics – Reproducibility

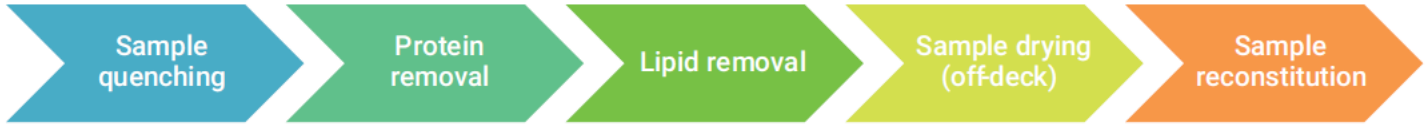


Figure 2. Metabolomics sample preparation workflow.

Figure 3. Agilent Captiva EMR–Lipid cartridges.

Methods to suit your needs

To get you started with this complex method development process, Agilent has a variety of liquid chromatography solutions for any skill level. These include: (1) the biocrates kits, (2) the ion-pairing dMRM solution, and (3) HILIC Z dMRM solution. A comparison of the different solutions is displayed in Table 1.

1. The **biocrates AbsoluteIDQ p180** (Figure 7) and **MxP Quant 500** (Figure 8) kits use the **Agilent 1290 Infinity II LC** and the Agilent **6470 triple quadrupole LC/MS**, and the **6495C triple** quadrupole LC/MS system, respectively, providing a simple, reproducible, and curated method by supplying consumables and software to get up and running quickly. These highly validated and easy-to-use kits provides basic metabolism and lipid information, including over 100 small molecules and 500 lipids.

2. With highly stable chromatography and high sensitivity, the ion-pairing **Metabolomics dMRM Database and Method** offers day-in and day-out performance for long sample runs. Using the **1290 Infinity II LC** and 6470 triple quadrupole LC/MS or **6495C triple** quadrupole LC/MS, this method provides good coverage of amino acids, the TCA cycle, and other energy metabolism pathways, with over 200 small molecules in the database. It must be noted that when using ion-pairing reagents, a dedicated LC system must be considered, as removing the ion-pairing reagents from the system is very challenging, and residual effects from reagents may linger for the lifetime of the LC.

3. The HILIC-Z dMRM method offers an extended coverage of core metabolic pathways and biological building blocks using the **Agilent 1290 Infinity II LC** or **1290 Infinity II bio LC system** coupled with the **6495C triple** quadrupole LC/MS. With over 400 small molecules in the database, this method offers more comprehensive biological information without the use of ion-pairing reagents. The **Agilent InfinityLab Poroshell 120 HILIC-Z** column allows for superior retention of polar metabolites using MS-compatible solvents, but also requires chromatographic expertise and the ability to follow method details exactly.

Figure 7. The Biocrates AbsoluteIDQ p180 kit.

Figure 8. The Biocrates MxP Quant 500 kit.

Sample Type Preparation Protocols - *20+ years of protocol development*

NIH Plasma Standards – Method Development

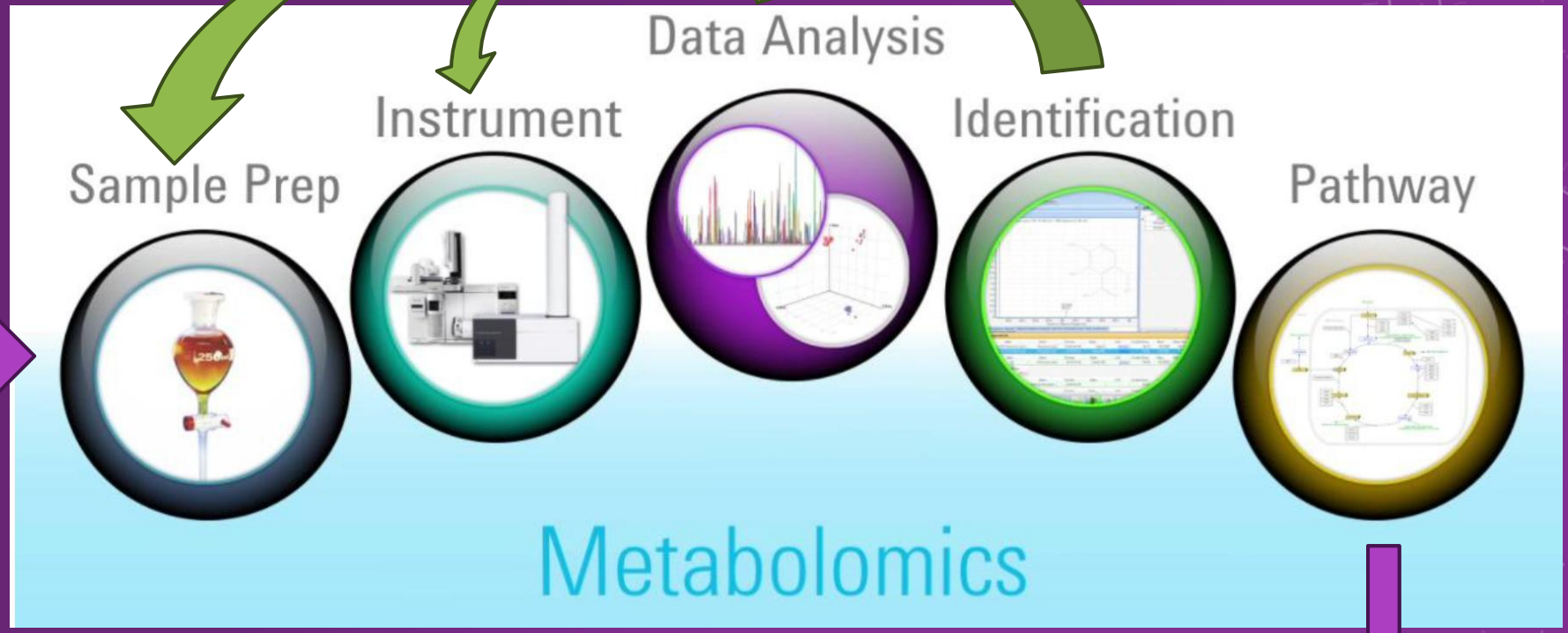
NIST® SRM® 1950

Website – Click Here

General Metabolomic Workflow and Drug Discovery

Bioanalysis view with the LC MS Metabolomics Workflow

Method Development



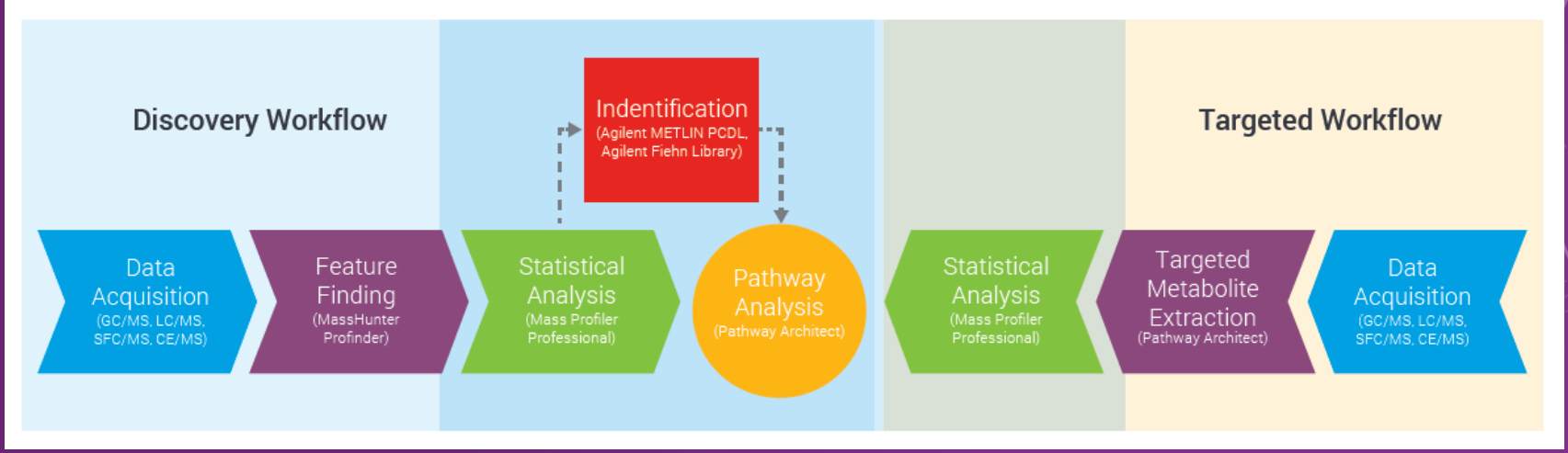
Communication between labs is important to success

Ask the Question(s) and Design the Experiment Workflow

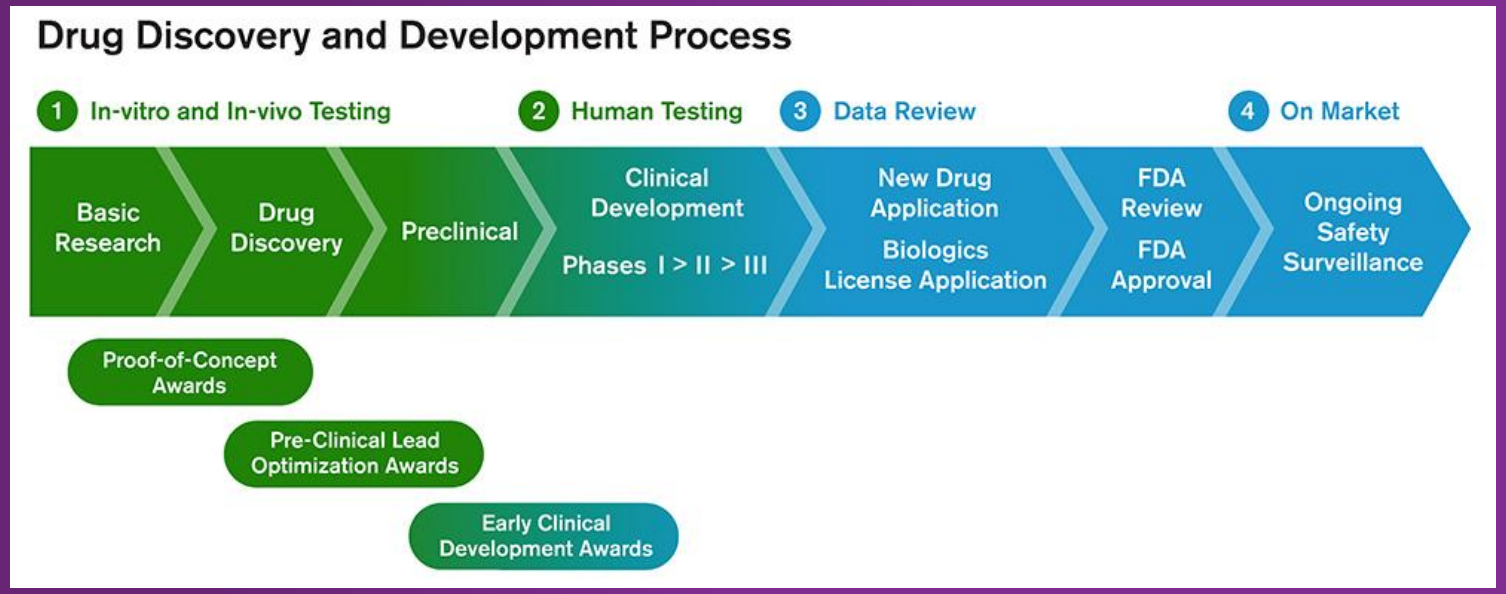
Answers to The Questions Asked?

Move to Next step of design

Discovery and Targeted Workflows for Metabolomics



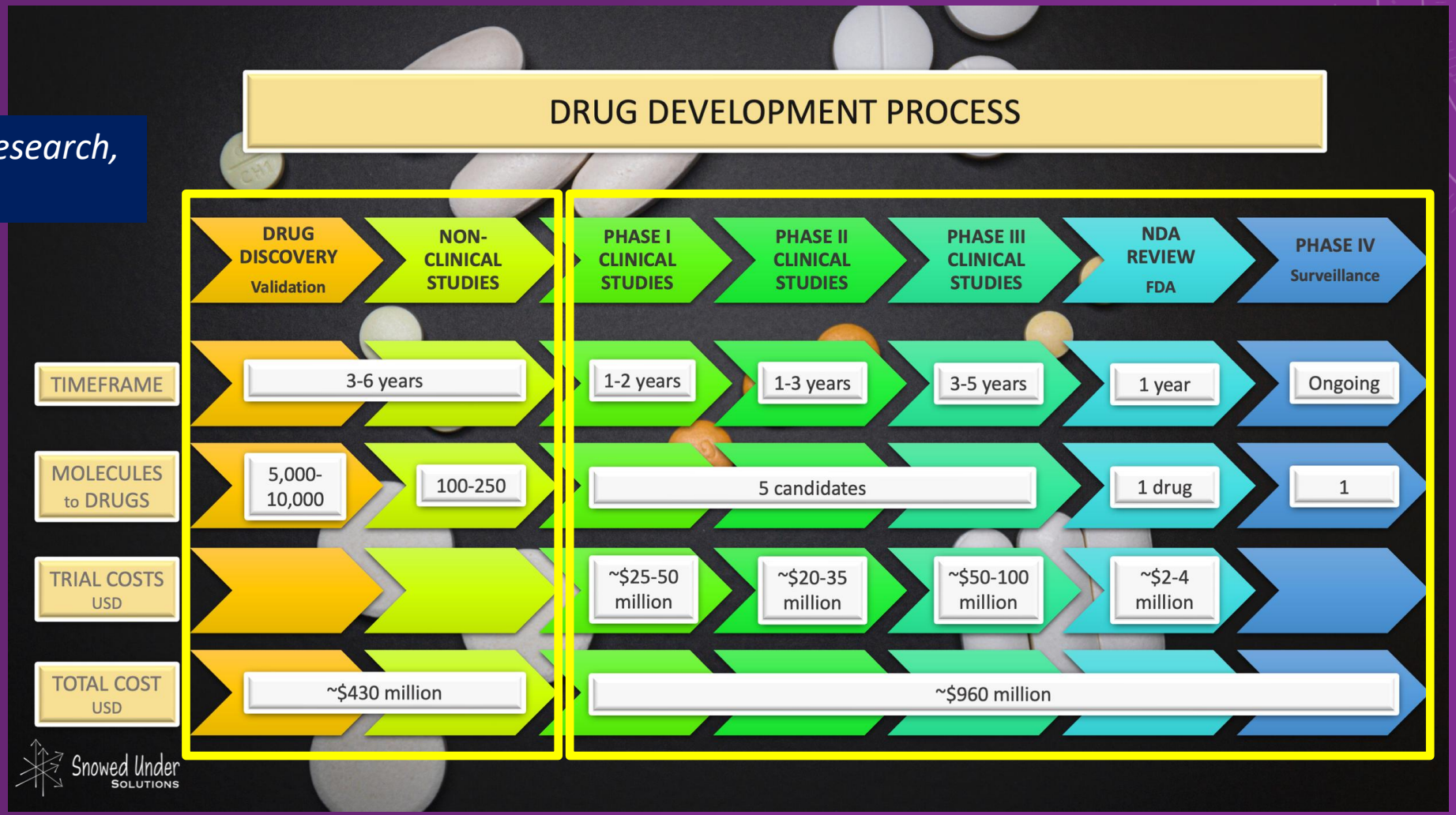
Relate to the Overall Drug Discovery Workflow



Discovery and Targeted Workflows for Metabolomics

Relate to the Overall Drug Discovery Workflow

It's called research, not search.



Where are we in the drug discovery process?

Think in a longitudinal mindset – what is the answer provided from my small molecule measurements

“Metabolomics in the clinical research laboratory is driven by understanding biological process and their relevance to understanding qualitative and quantitative clinical data and outcomes.

For example, a clinical metabolite panel of catecholamines may focus on quantitation of dopamine, epinephrine, and norepinephrine: the three most common biomarkers of stress.

However, in a translational laboratory, the focus would be on the larger biosynthetic pathway for catecholamines to understand the underlying production of the neurotransmitters, their interactions, and their mechanistic relationships as a marker of stress.

Analytical challenges in a clinical research or translational laboratory are ultimately driven by the biological process and clinical chemistry, which need to be understood. Knowledge of not only the chemistry of the clinical panel, but a broader perspective of metabolism is required to help solve analytical challenges and maximize study return of investment.”

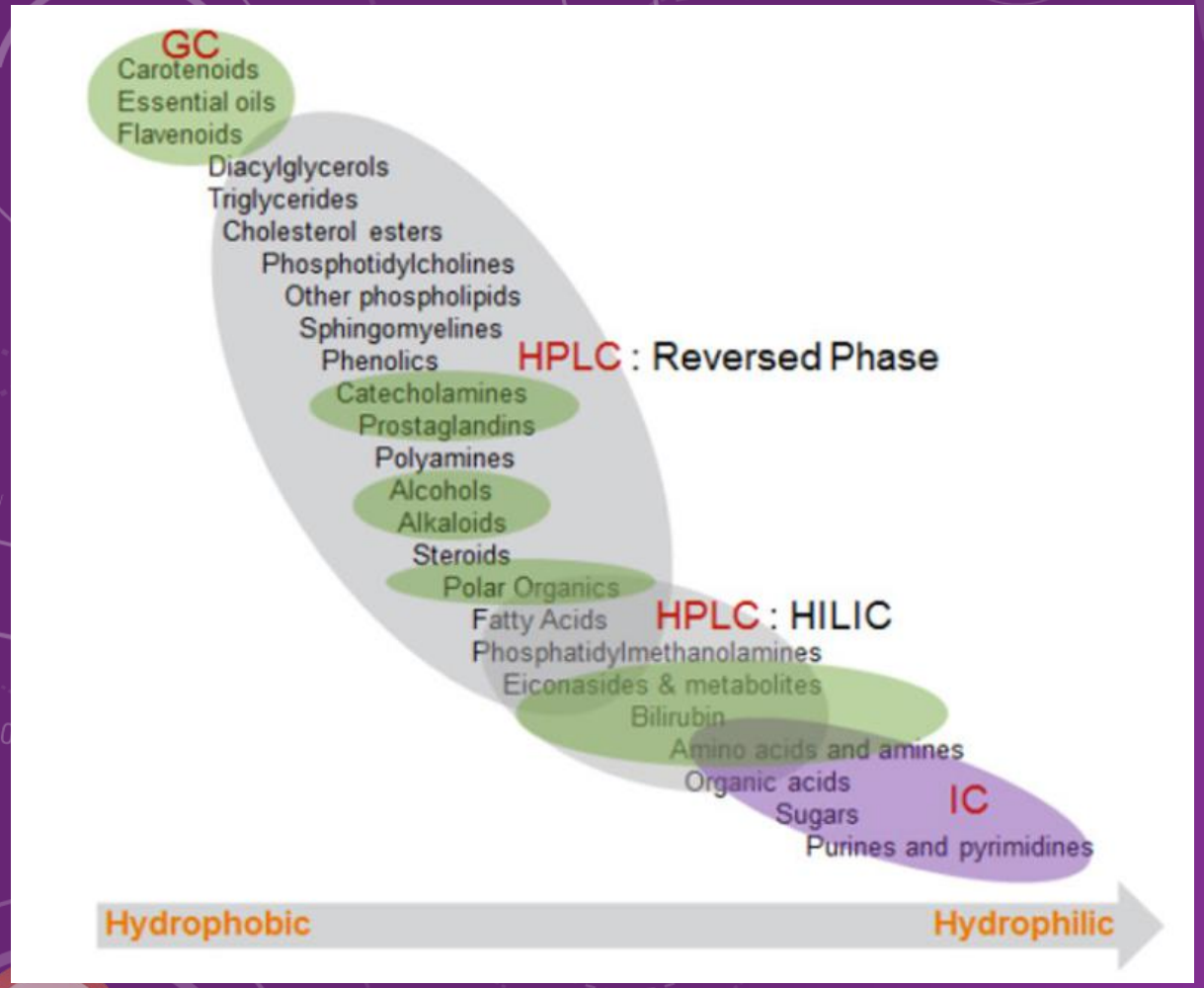
Chromatography - The focal point of experimental design

What is molecular class of interest? (Targeted)
Mapping Metabolomic Profile of System? (Global)
Mechanism of Action Study based on Drug Treatment and Time Points?

Longitudinal Study? – Lock in methods first for downstream experiments
Movement from ***Discovery*** *to* ***Translational*** *to* ***Diagnostic***

Bioanalysis View – Match the Molecular System to the Chromatography Method(s)

Liquid Chromatography and *Multiple Experiment Types*



Choice in Chromatography and Method

- Reversed Phase C18 Most Common

- Common Gradients to need of experiment
  i.e., 3, 5, 15, 25, etc. minute gradients.

- Discovery or Profile Metabolomics.
  Perform several "experiments" on the same sample for full LCMS *metabolomic profile*
  i.e. RP Chrom (+/-)
  or NP/HILIC (+/- modes)

- More Targeted Pathway and/or class of molecules
  Perform single, specific method for molecules of choice

- *Molecules in pathways move together*

Instrument, Measurement, and Data Analysis

Mass Spectrometry Measurement - Why the 6546 LCQ-TOF?

Accurate mass measurement improves several quality attributes of the measurement for informatics.

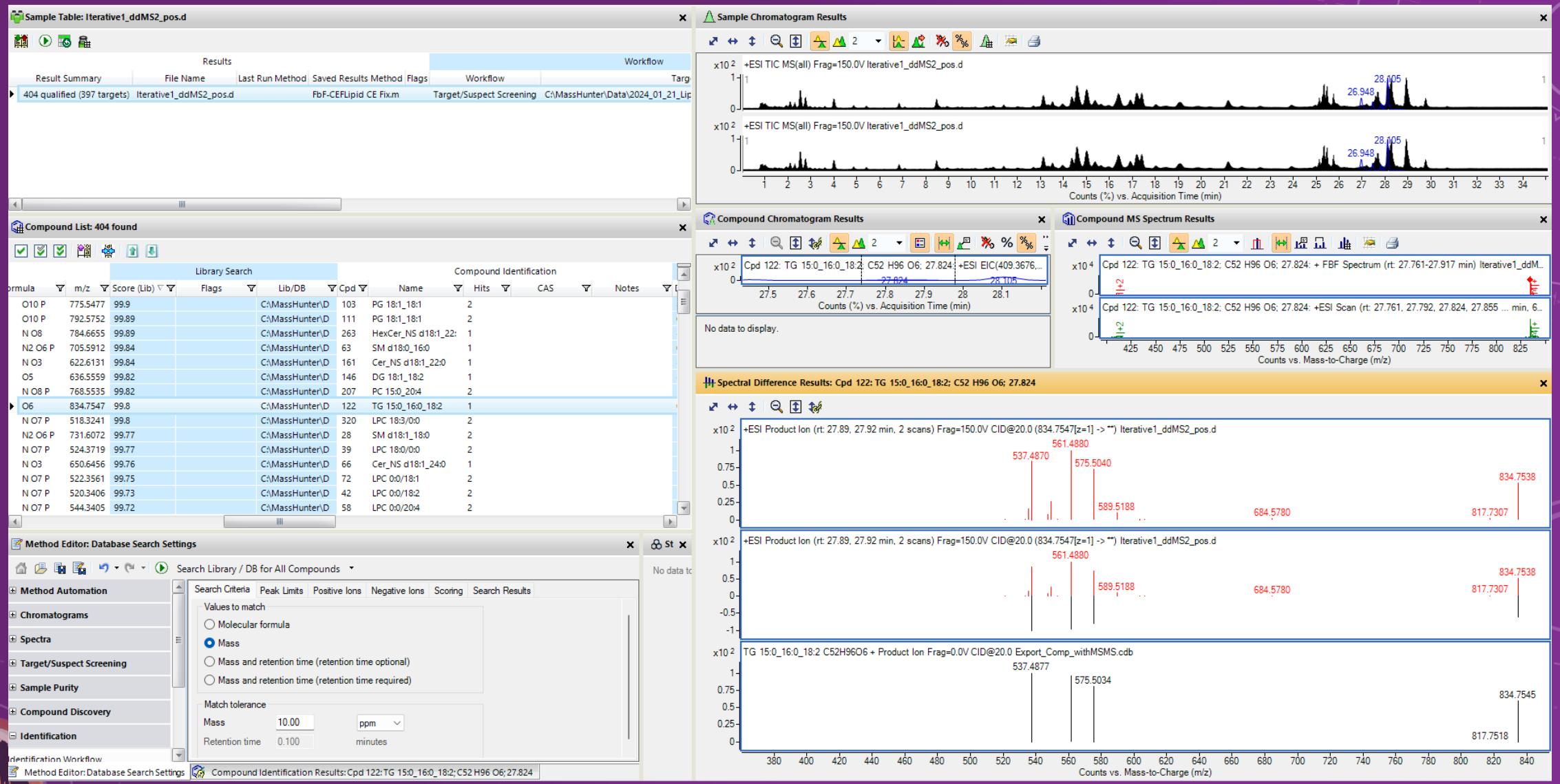
Rapid peak extraction for informatics.

Reduce the number of possible answers for small molecule identification.

Resolving power for coeluting species.

5x in scan dynamic range.

Agilent Packages of Software for Rapid Data Extraction and Analysis



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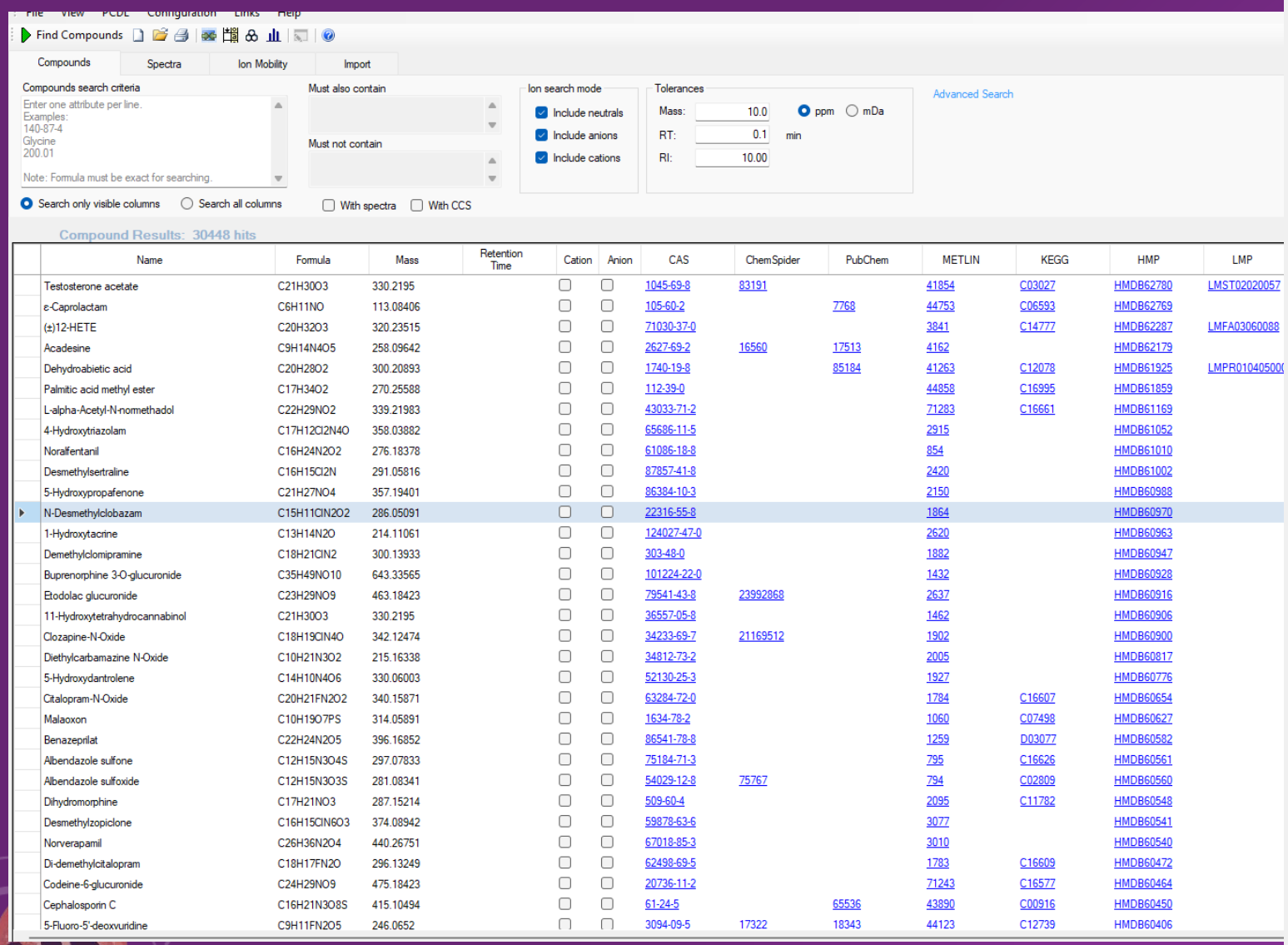
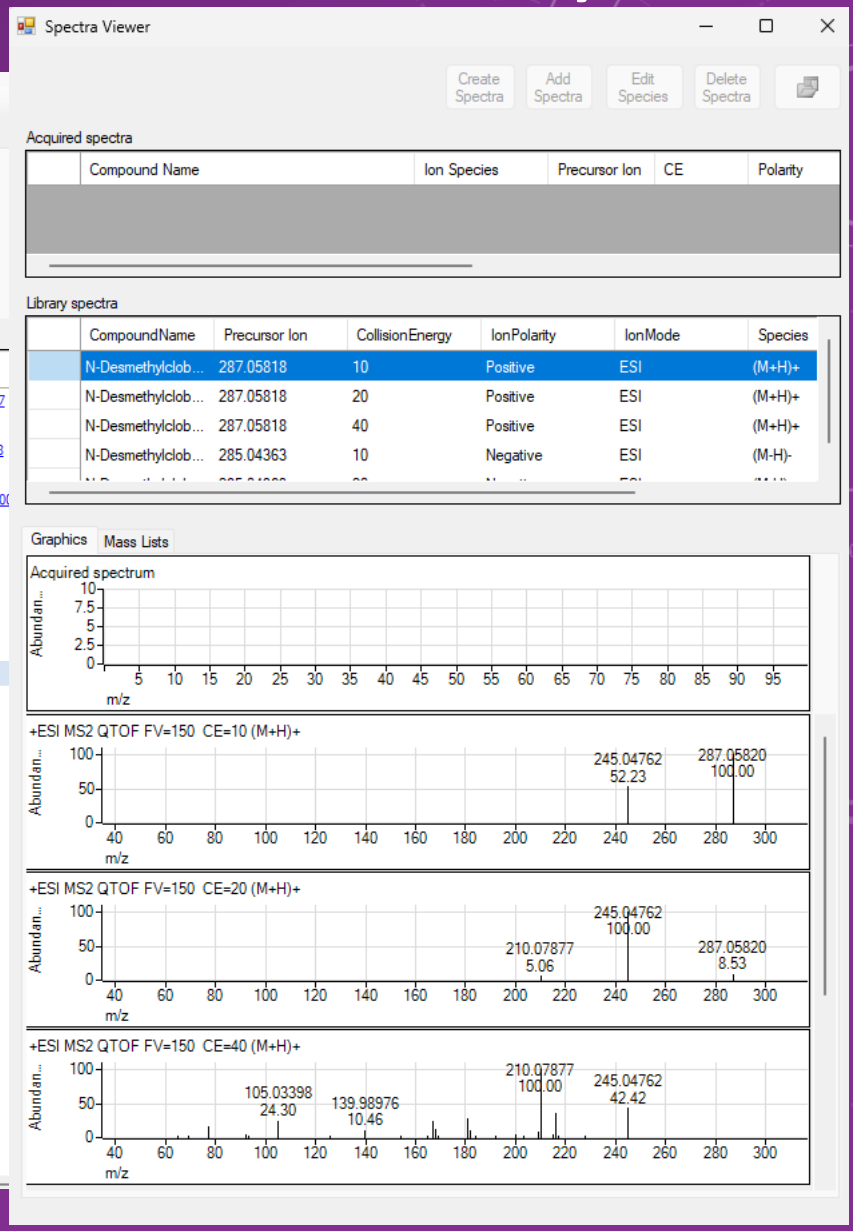


Name	Formula	Mass	Retention Time	Cation	Anion	CAS	ChemSpider	PubChem	METLIN	KEGG	HMP	LMP
Testosterone acetate	C21H30O3	330.2195				1045-69-8	83191		41854	C03027	HMDB62780	LMST02020057
ε-Caprolactam	C6H11NO	113.08406				105-60-2		7768	44753	C06593	HMDB62769	
(±)12-HETE	C20H32O3	320.23515				71030-37-0			3841	C14777	HMDB62287	LMFA03060088
Acadesine	C9H14N4O5	258.09642				2627-69-2	16560	17513	4162		HMDB62179	
Dehydroabietic acid	C20H28O2	300.20893				1740-19-8		85184	41263	C12078	HMDB61925	LMPR01040500
Palmitic acid methyl ester	C17H34O2	270.25588				112-39-0			44858	C16995	HMDB61859	
L-alpha-Acetyl-N-normethadol	C22H29NO2	339.21983				43033-71-2			71283	C16661	HMDB61169	
4-Hydroxytriazolam	C17H12Cl2N4O	358.03882				65686-11-5			2915		HMDB61052	
Noralfentanil	C16H24N2O2	276.18378				61086-18-8			854		HMDB61010	
Desmethylsertraline	C16H15Cl2N	291.05816				87857-41-8			2420		HMDB61002	
5-Hydroxypropafenone	C21H27NO4	357.19401				86384-10-3			2150		HMDB60988	
N-Desmethylclobazam	C15H11ClN2O2	286.05091				22316-55-8			1864		HMDB60970	
1-Hydroxytacrine	C13H14N2O	214.11061				124027-47-0			2620		HMDB60963	
Demethylclomipramine	C18H21ClN2	300.13933				303-48-0			1882		HMDB60947	
Buprenorphine 3-O-glucuronide	C35H49NO10	643.33565				101224-22-0			1432		HMDB60928	
Etodolac glucuronide	C23H29NO9	463.18423				79541-43-8	2392868		2637		HMDB60916	
11-Hydroxytetrahydrocannabinol	C21H30O3	330.2195				36557-05-8			1462		HMDB60906	
Clozapine-N-Oxide	C18H19ClN4O	342.12474				34233-69-7	21169512		1902		HMDB60900	
Diethylcarbamazine N-Oxide	C10H21N3O2	215.16338				34812-73-2			2005		HMDB60817	
5-Hydroxydantrolene	C14H10N4O6	330.06003				52130-25-3			1927		HMDB60776	
Citalopram-N-Oxide	C20H21FN2O2	340.15871				63284-72-0			1784	C16607	HMDB60654	
Malaoxon	C10H19O7PS	314.05891				1634-78-2			1060	C07498	HMDB60627	
Benazeprilat	C22H24N2O5	396.16852				86541-78-8			1259	D03077	HMDB60582	
Albendazole sulfone	C12H15N3O4S	297.07833				75184-71-3			795	C16626	HMDB60561	
Albendazole sulfoxide	C12H15N3O3S	281.08341				54029-12-8	75767		794	C02809	HMDB60560	
Dihydromorphine	C17H21NO3	287.15214				509-60-4			2095	C11782	HMDB60548	
Desmethylzopiclone	C16H15ClN6O3	374.08942				59878-63-6			3077		HMDB60541	
Norverapamil	C26H36N2O4	440.26751				67018-85-3			3010		HMDB60540	
Di-demethylcitalopram	C18H17FN2O	296.13249				62498-69-5			1783	C16609	HMDB60472	
Codeine-6-glucuronide	C24H29NO9	475.18423				20736-11-2			71243	C16577	HMDB60464	
Cephalosporin C	C16H21N3O8S	415.10494				61-24-5		65536	43890	C00916	HMDB60450	
5-Fluoro-5'-deoxyuridine	C9H11FN2O5	246.0652				3094-09-5	17322	18343	44123	C12739	HMDB60406	



For Life Sciences Research Only, Not for Diagnostic Purposes

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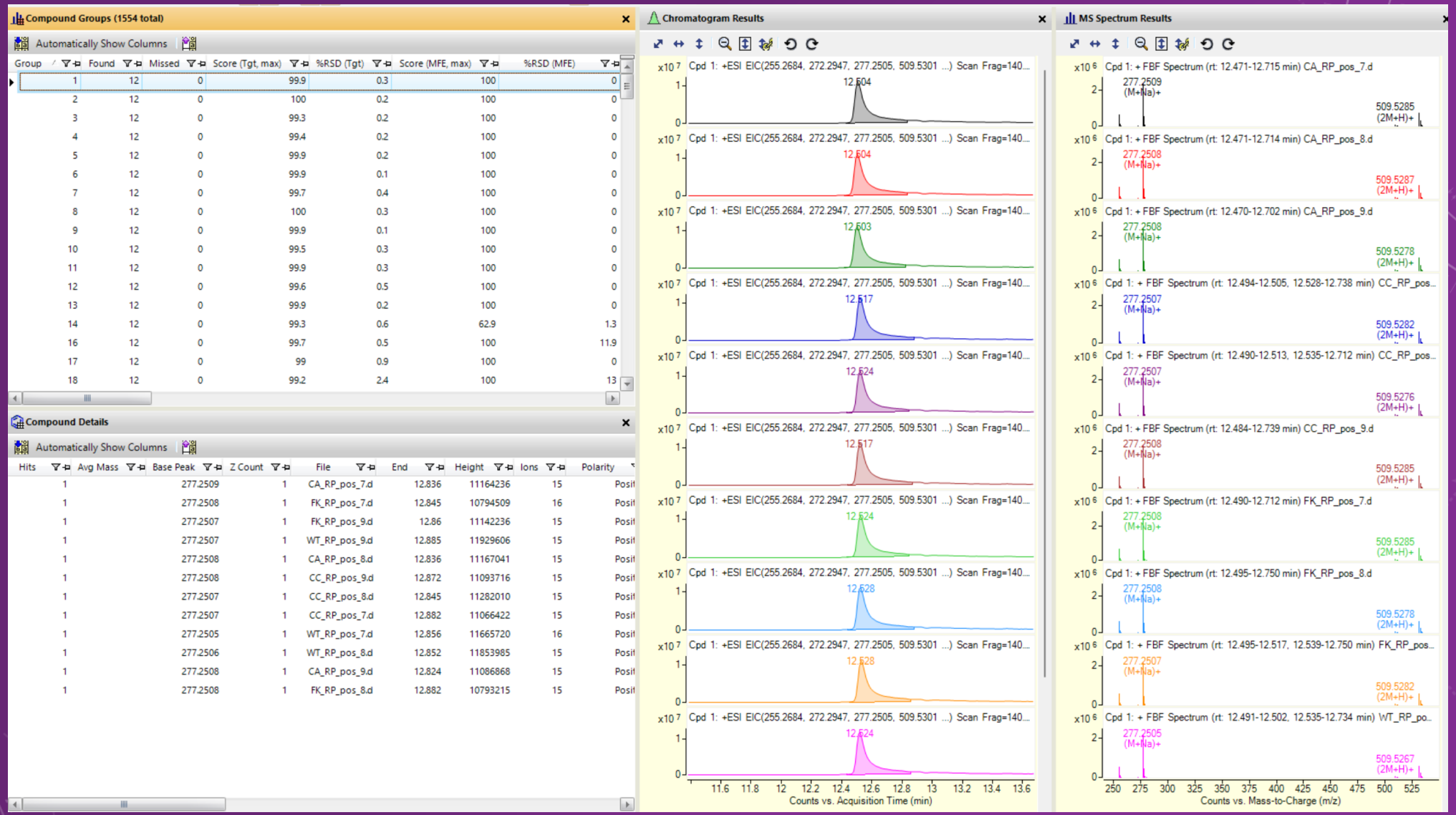

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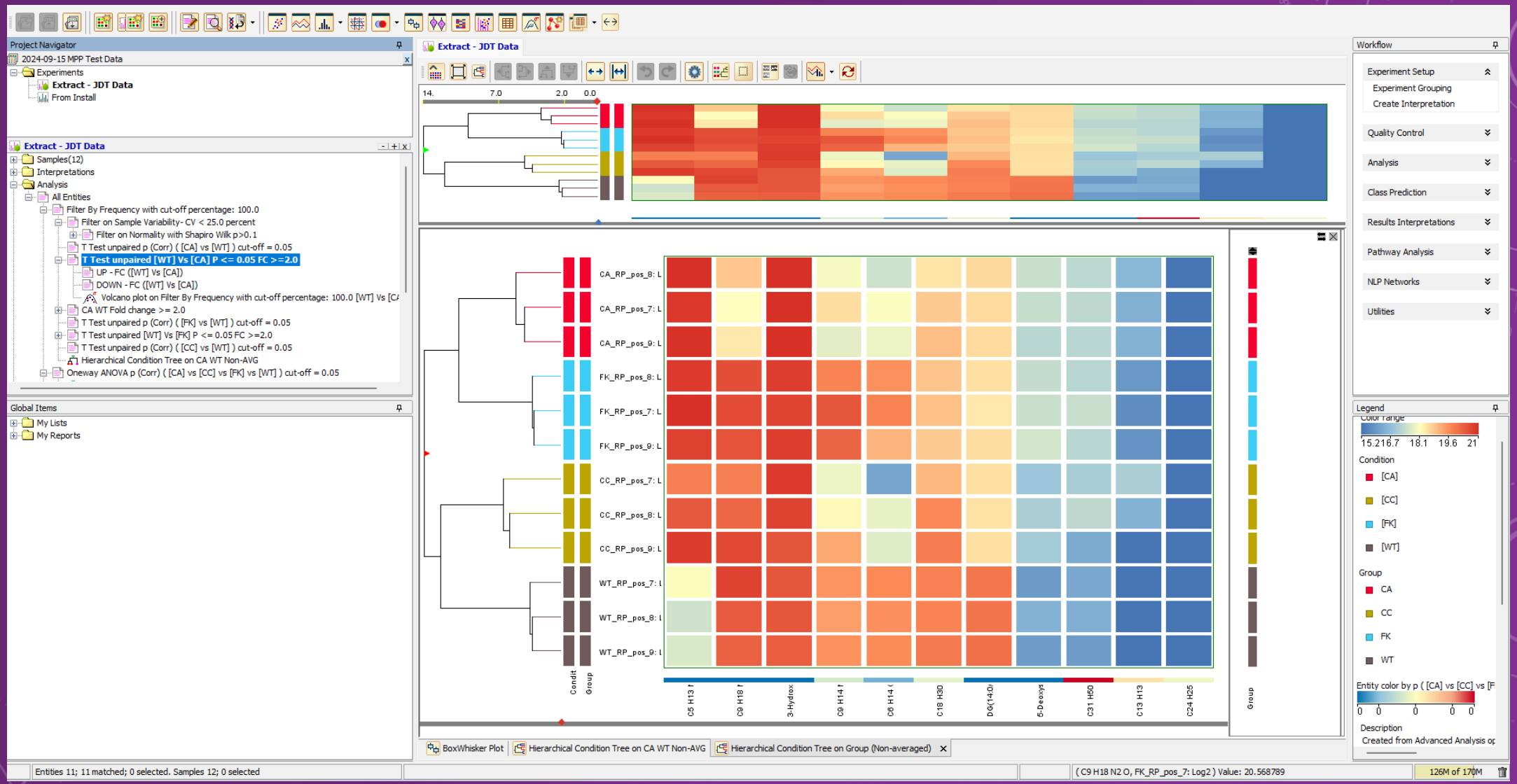


For Life Sciences Research Only, Not for Diagnostic Purposes

Applications and Examples – With Agilent QTOF Technologies

Sum = composition

Sum = concentration

Application Note

Lipidomics

Agilent

Trusted Answers

Lipid Profiling Workflow Demonstrates Disrupted Lipogenesis Induced with Drug Treatment in Leukemia Cells

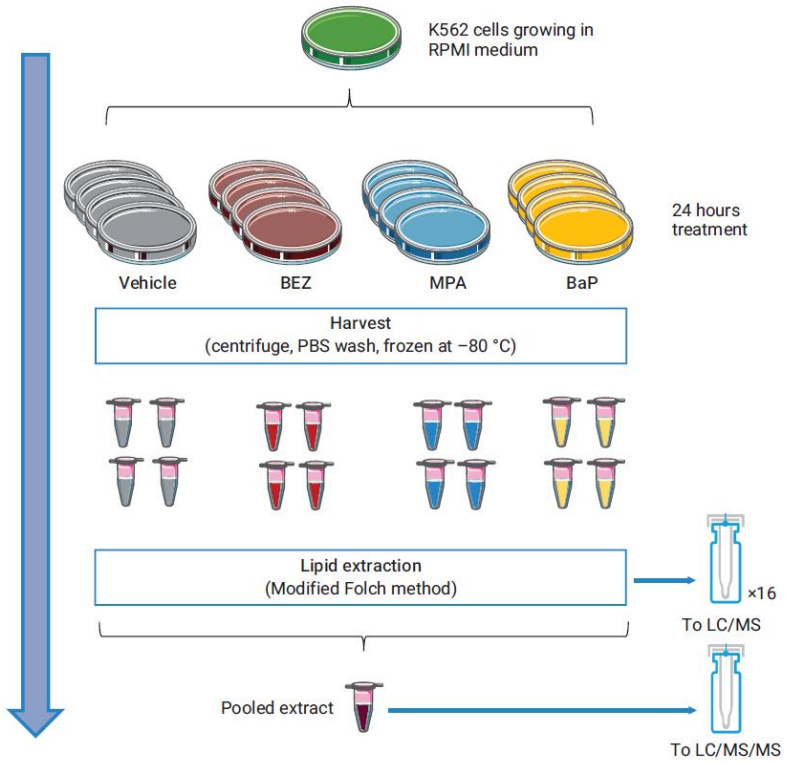
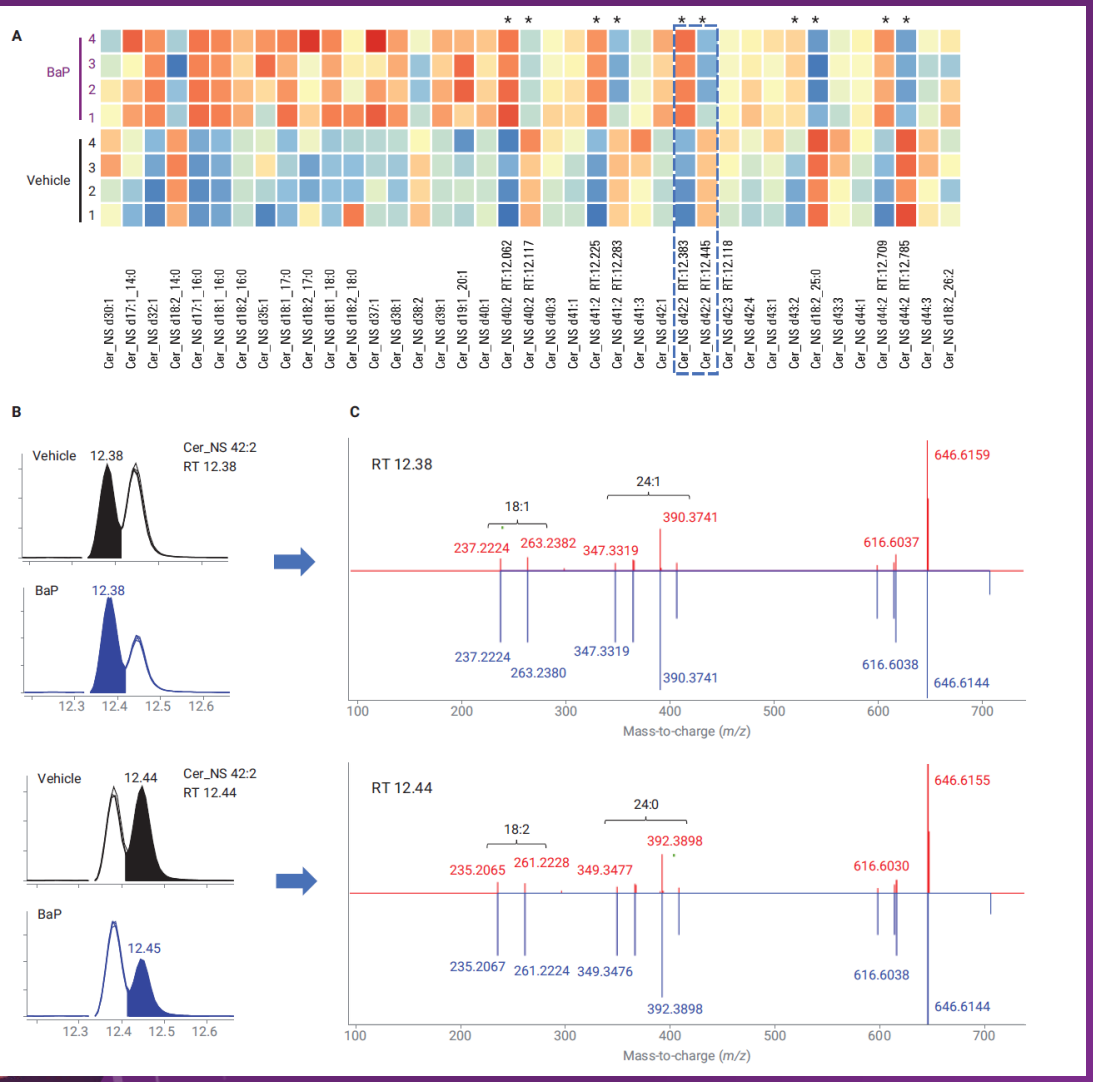


Figure 1. Experimental design for studying effects of drug treatments on cancer cell lipidome.

20 + years of Metabolomics Research and Method Development

Resolve Isobars with Chromatography (RP C18)
Match to MSMS Library (Lipid Annotator Supported)



Specific Changes Visualized

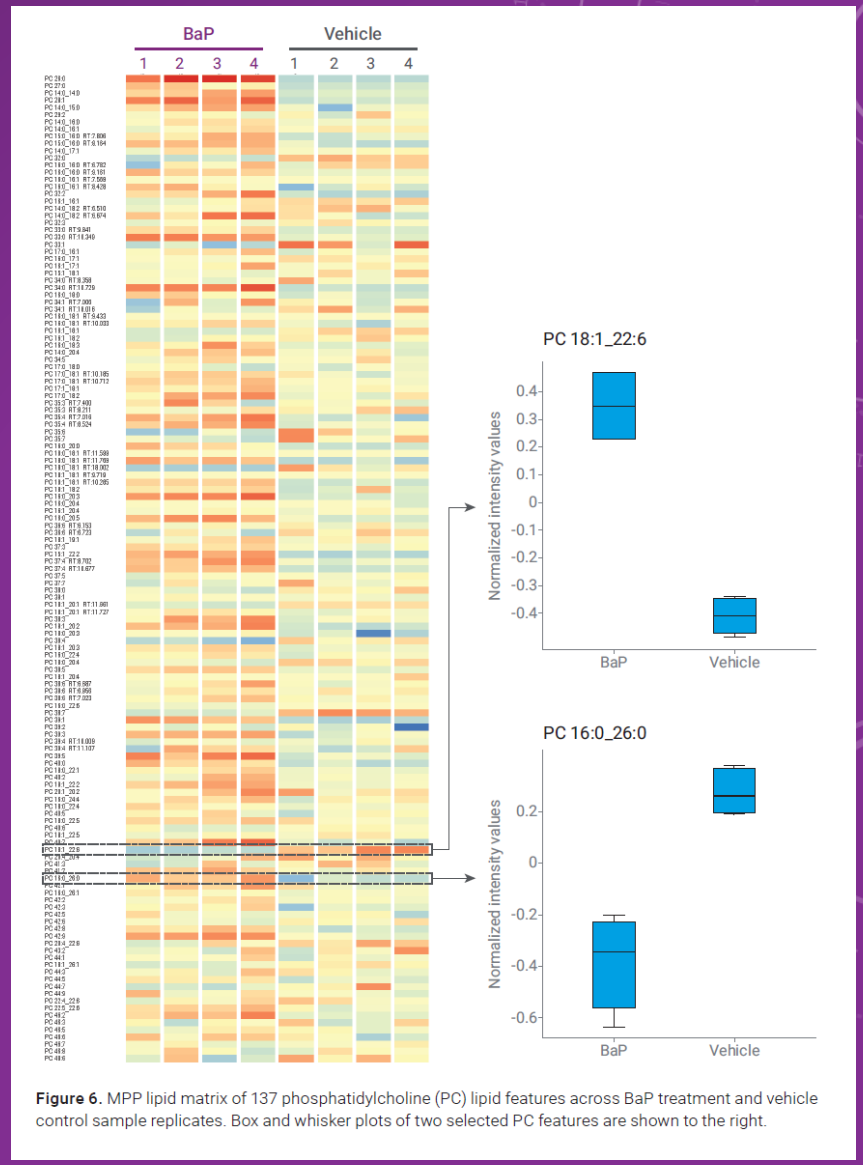


Figure 6. MPP lipid matrix of 137 phosphatidylcholine (PC) lipid features across BaP treatment and vehicle control sample replicates. Box and whisker plots of two selected PC features are shown to the right.

Lipid Class Analysis and Relative Quantitation of Global Data (How you use the data)

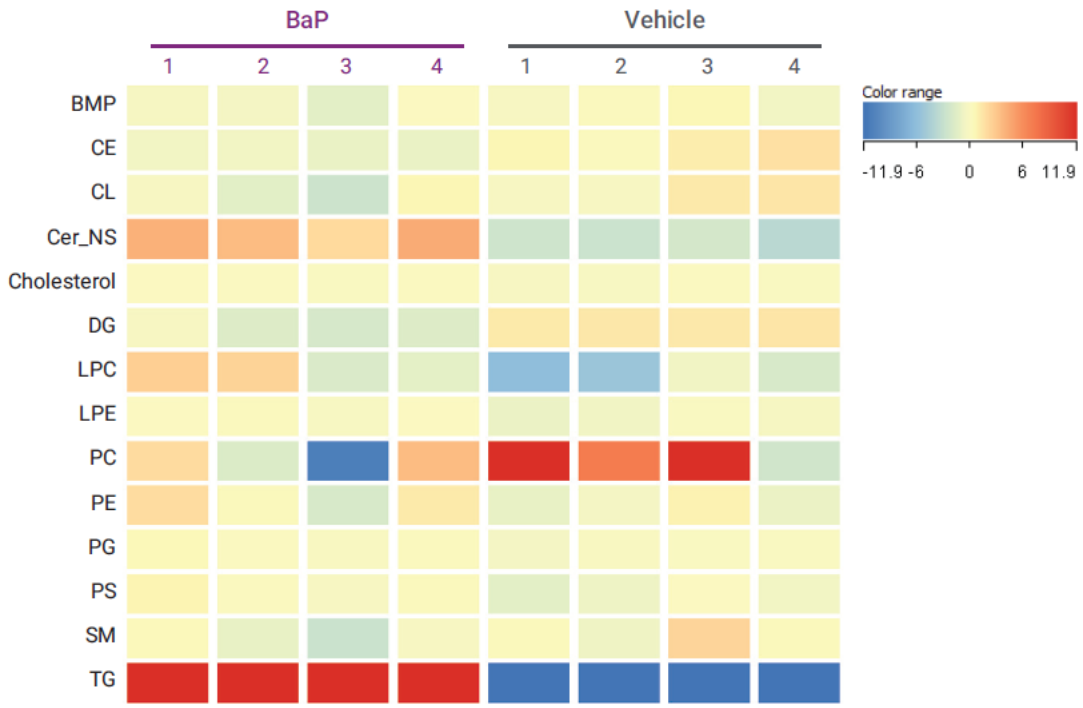


Figure 5. MPP lipid class matrix of total normalized lipid class abundances across BaP treatment and vehicle control sample replicates. The color range represents the sum of normalized, transformed abundances for all lipid features within a lipid class.

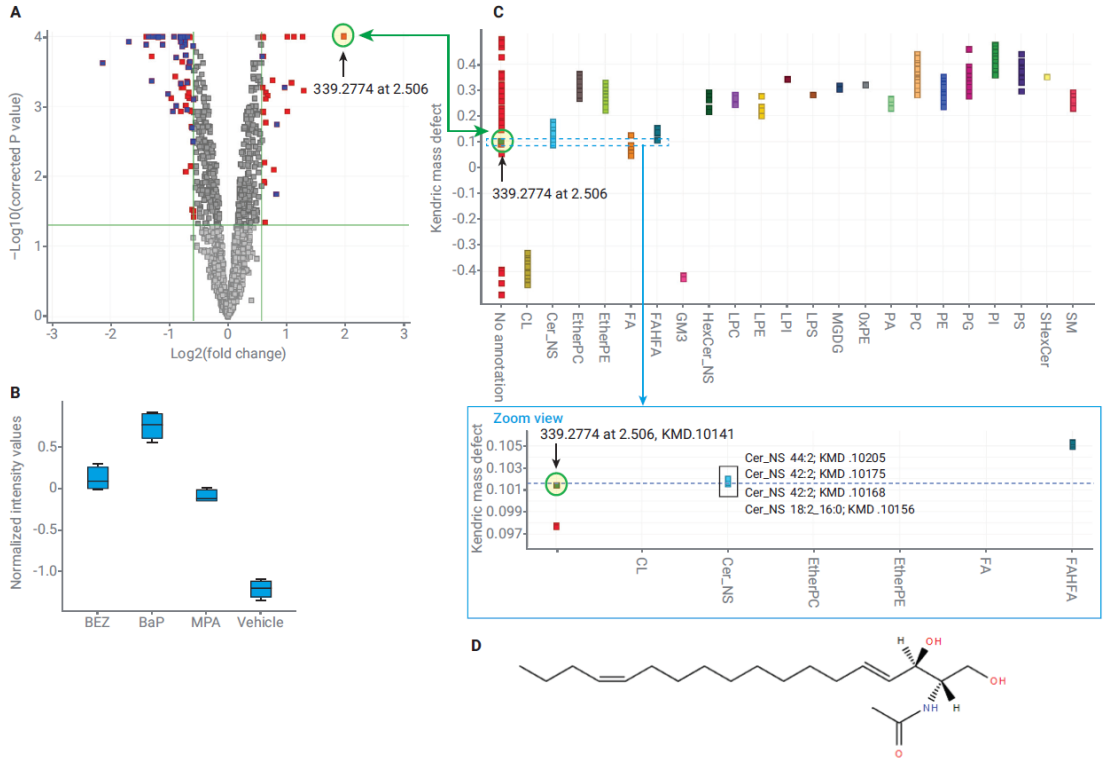


Figure 8. Elucidation of an unknown differential feature with the untargeted workflow. (A) MPP Volcano plot from a moderated t-test with Benjamini-Hochberg FDR multiple testing correction for BaP treatment versus vehicle control. Significant features (fold change cutoff >1.5, p-value >0.05) are colored in blue (annotated lipids) and red (unannotated features). The feature of interest (*m/z* 339.2774 at 2.506) is circled in green. (B) Box and whisker plot of the feature of interest for the four drug treatment conditions. (C) MPP Kendrick mass defect (KMD) plot for the combined entity list of 513 annotated lipids with the list of 93 differential features (n = 565). Features that could not be annotated with the PCDL are shown in the first column in red. The zoomed region shows the alignment by KMD of the feature of interest with a group of Cer_NS lipids. (D) Cer_NS 18:2_2:0 candidate structure for the feature of interest.

Impact of Chromatography on Lipid Profiling of Liver Tissue Extracts

2015

Note Classes

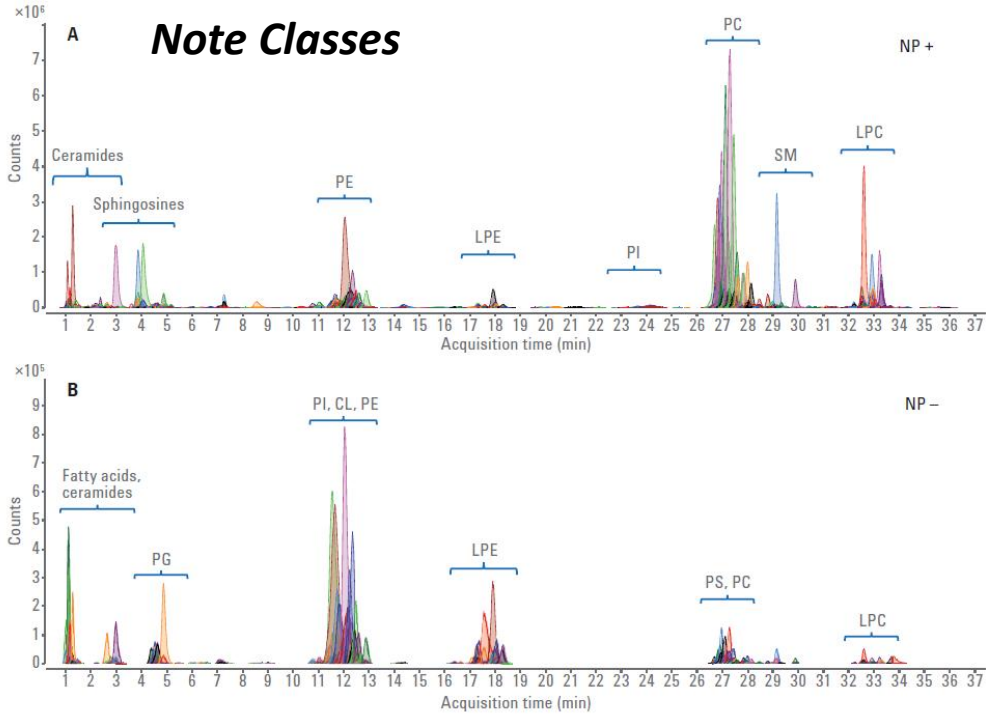
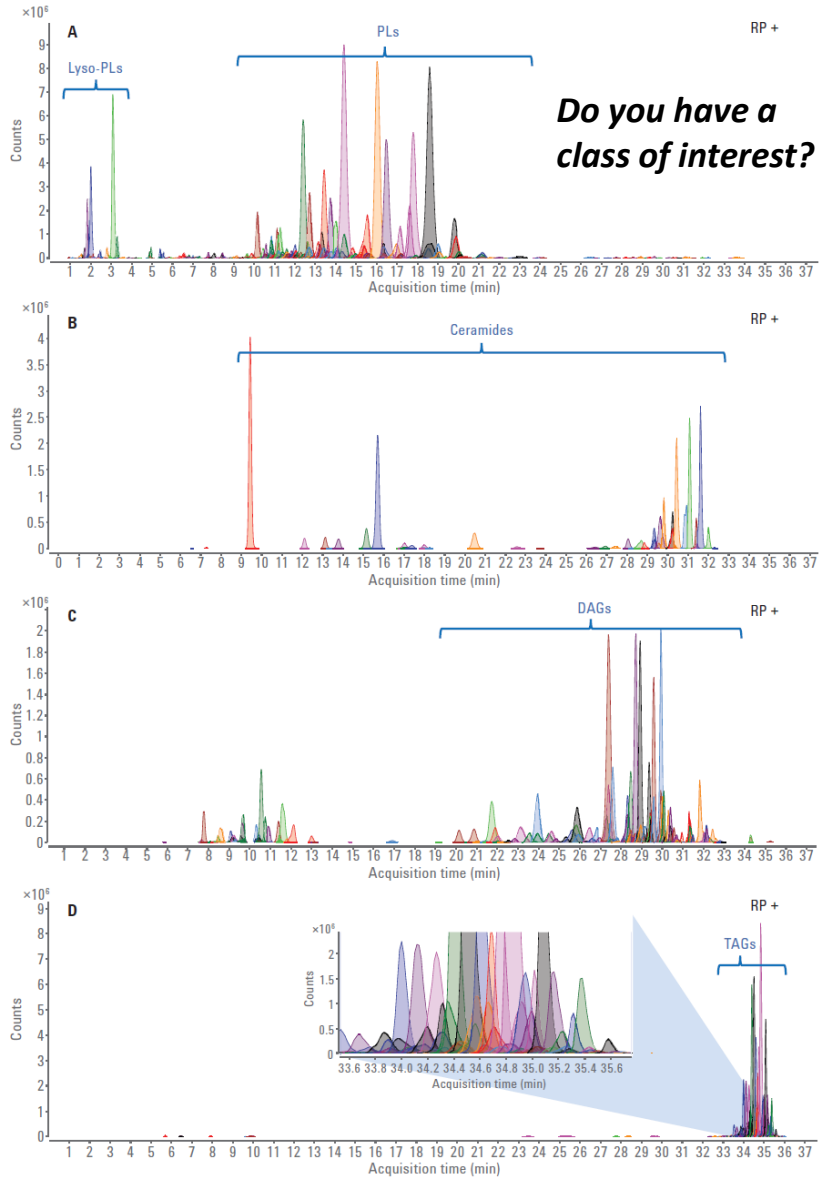


Figure 3. Elution profiles of lipid classes from a liver lipid extract resolved with NP chromatography and analyzed in positive and negative ion modes. CL: cardiolipin; LPC: lysophosphatidylcholine; LPE: lysophosphatidylethanolamine; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PG: phosphatidylglycerol; PI: phosphatidylinositol; PS: phosphatidylserine; SM: sphingomyelin.

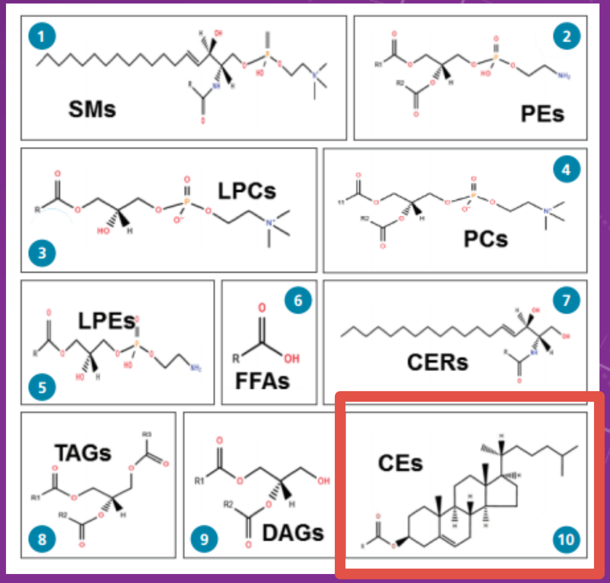
Do you have a class of interest?

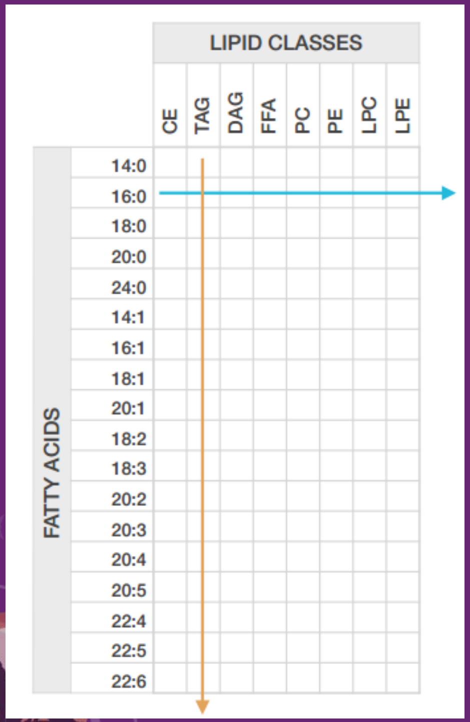


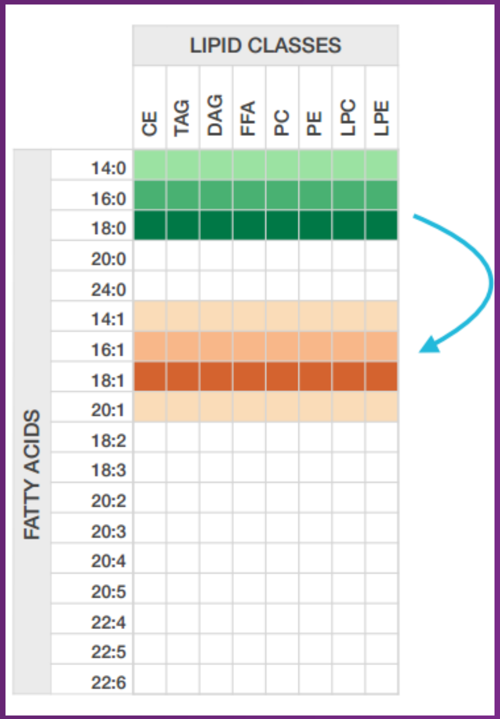
Lipids – Specific subclass of metabolomics – because of molecule class properties and extraction conditions
Bioanalysis View

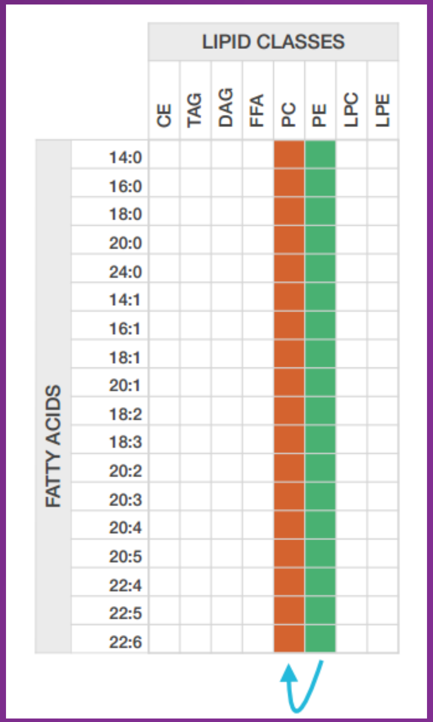
Lipid are present in classes that have concentrations and compositions (important for level of metabolism)

- Concentration = sum of the FAs for any given class (column)
- Composition









When FA metabolism is altered there is the ability to change FA composition of all classes

When lipid class metabolism is altered there is the ability to change all members of the class

Quantitative Determination of a Panel of Endogenous Steroids in Human Serum by LC/MS/MS

Using an Agilent Supported Liquid Extraction (SLE) Chem Elut S Plate

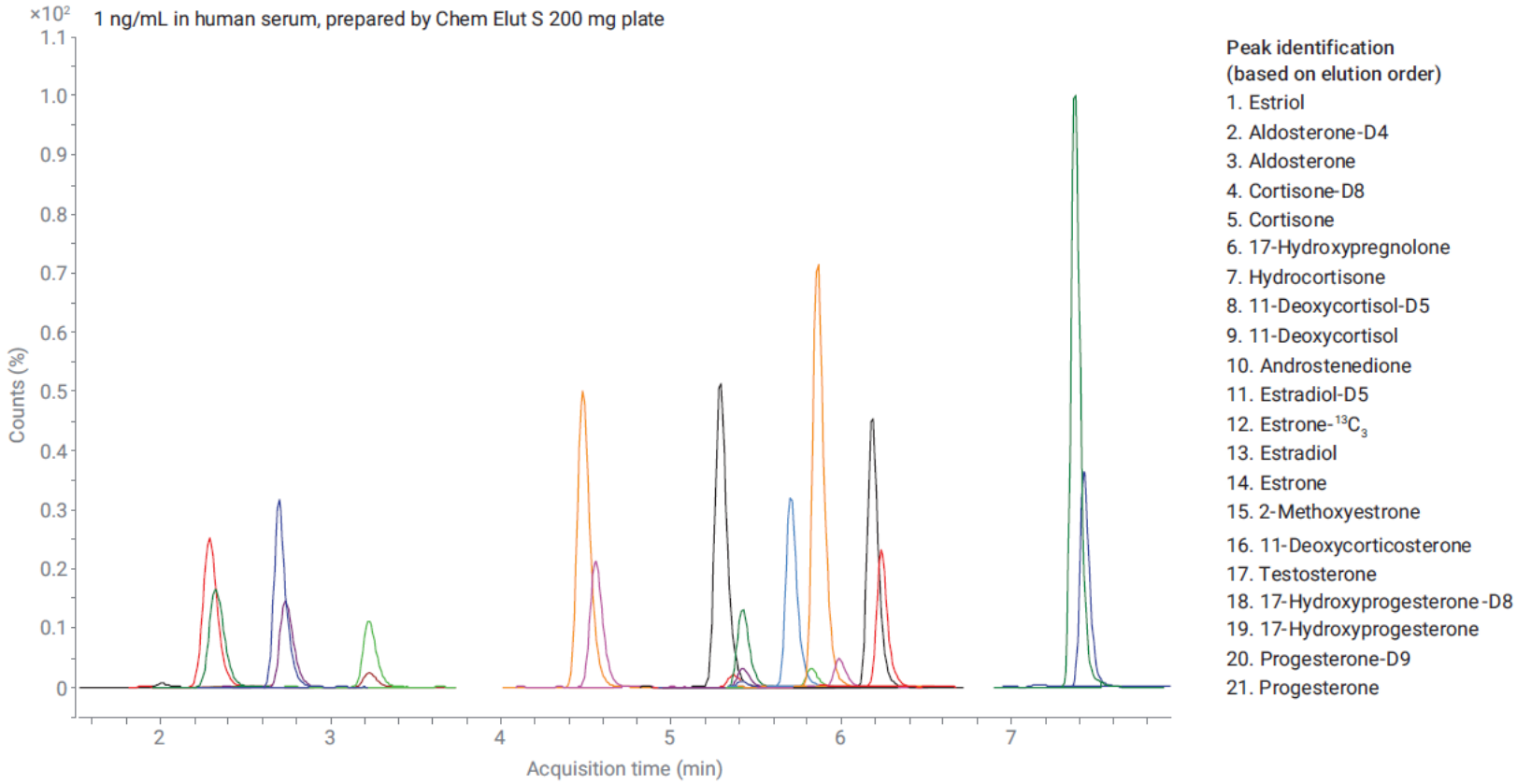


Figure 2. An LC/MS/MS chromatogram of 1 ng/mL steroids in serum prepared using the Agilent Chem Elut S supported liquid extraction method.

Integrate metabolomics with other omics

While genomics, transcriptomics, proteomics, and metabolomics are in wide use in both industry and academia, these experiments—performed alone—are often insufficient to uncover meaningful correlations amid the high level of noise omics experiments typically generate. Integration of data from multiple omics can provide enough constraints to greatly improve the signal-to-noise of the analysis. The Pathway Architect module of Mass Profiler Professional allows either single omics analysis or joint analysis of multiple omics, enabling you to discover commonly affected pathways and aid in your ability to find reliable answers more quickly.

Gene Expression

GeneSpring GX

Agilent's GeneSpring provides powerful, accessible statistical tools for intuitive data analysis and visualization. Designed specifically for the needs of biologists, GeneSpring offers an interactive environment that promotes investigation and enables understanding of Transcriptomics, Genomics, Metabolomics, Proteomics and NGS data within a biological context.

GeneSpring allows you to quickly and reliably identify targets of interest that are both statistically and biologically meaningful. For Research Use Only.

Not for use in diagnostic procedures.

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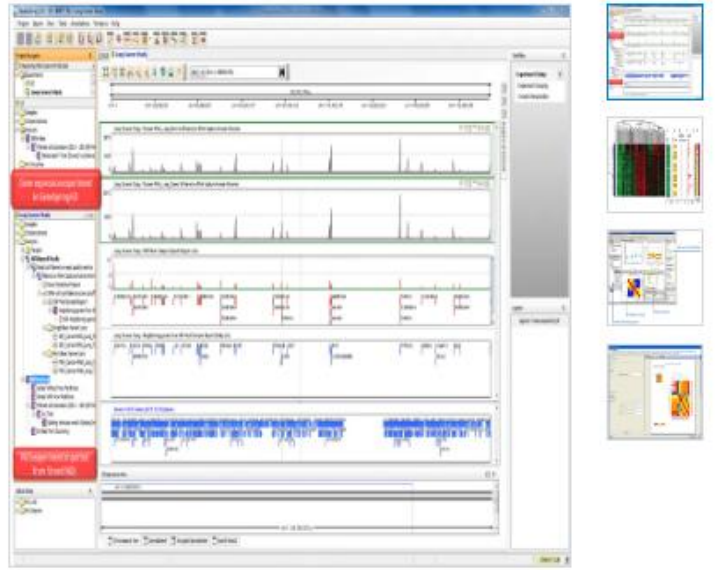

Thank you

Move into Q&A Session