Standard Assays Offered by the Lipomics Laboratory.

- **Lipid extraction.** Lipids from tissue (5-10 mg), blood plasma (100 – 200 µl) or cells (1 x10^6 – 5 x10^6) are generally extracted utilizing the method of Bligh and Dyer [13] or solvents developed for specific classes of lipids.

- **Fatty acid composition of lipid subfractions.** Separation of lipids into different neutral and phospholipid class is achieved by thin layer or reverse phase chromatography. Authentic C17-containing standard lipids are spiked into samples and co-chromatographed to identify the lipid subfractions and to estimate lipid recovery. Protocols for other forms of thin-layer chromatography (TLC), viz., two dimensional TLC, argentation TLC (for separation according to the number of double bonds), boric acid impregnated TLC (for isomeric diol containing lipids) are also routinely used in the Laboratory. Cardiolipid, sphingolipid fatty acid quantitation is also available. Methyl ester containing 10 to 28 carbon atoms with various degrees of unsaturation can be quantified.

- **Trans fatty acids from plasma or adipose tissue.** Trans fatty acids in the total lipid choloroform extracts or in total free fatty acid pool hexane extracts are esterified with boron trifluoride-methanol reagent. The isolated methyl esters in hexane are dried under nitrogen gas, redissolved in a small volume of hexane and then analyzed by GC with a 100 m fused silica capillary cis/trans column. A known amount of authentic C17-fatty acid standard is used for quantification utilizing calibration curve for each fatty acid.

- **Acyl CoA and ceramides.** Tissue samples (30-50 mg) are extracted with saturated ammonium sulfate and acetonitrile, and further purified by extraction on an oligonucleotide purification cartridge and elution with 60% acetonitrile. Samples are dried, resuspended and subjected to GC/MS or ESI/MS/MS analysis. Quantification is provided by comparison to standard compounds[14].

- **Acyl carnitine by GC/MS or LC/ESI/MS/MS.** Lipids are extracted with acetonitrile (100%) from plasma or tissues of interest and extracts are supplemented with ^2^H_9_free carnitine, ^2^H_3_acetyl carnitine (C2:0), ^4^H_3-propionyl carnitine (C3:0), ^4^H_3-butyryl carnitine (C4:0), ^4^H_3-isovaleryl carnitine (C5:0), ^4^H_3-octanoyl carnitine (C8:0), ^4^H_3-myristoyl carnitine (C14:0) and ^4^H_3-palmitoyl carnitine (C16:0) as internal standard mixture. Acylcarnitines are subsequently analyzed by LC/ESI/MS/MS. Extracted ion chromatograms show that the most abundant peak is the neutral loss to m/z 85 which used for quantification.

- **Phospholipids.** Assays for a variety of phospholipids have been developed including: phosphotidylinositols, phosphochoelines, phosphoserines, phosphatidic acid, cyclic phosphatidic acid employing LC/MS and GC/MS analysis after extraction.

- **Steroids.** The core has developed an procedure for the analysis of 9 different steroids (Cortisone, cortisol, corticosterone, 11-deoxycortisol, androstenedione, 11-deoxy cortisol, testosterone 17-OH progesterone and progesterone) in blood by a single LC/MS/MS run with a sensitivity in the 1-50 pg range and requiring 50-100 µL of plasma or serum. Separate assays are available for estradiol and estrone.

- **Quality Control.** Lipid extraction is generally performed by adding a known amount of heptadecanoyl (C17:0) fatty acid as internal standard lipid. A standard mixture of fatty acids ranging carbon chain from C14 up to C24, containing both saturated and unsaturated and isomeric double bond position are analyzed with each sample run to monitor the separation pattern of fatty acid methyl esters. A pool of a standard plasma sample is analyzed periodically by GC to determine the reproducibility of the quantifications with time. In general, the CV is < 5% for repeated measures. For tandem MS analysis of acyl CoA and acyl
carnitine, similar utilization of tissue standard is followed to ensure the reproducibility and quality of the analysis.

**Directed Metabolomics Laboratory.**

**Standard Assays Offered by the Directed Metabolomics Laboratory.**

- **Metabolite extraction.** The core lab works with investigators to implement appropriate sample preparation methodology, with the goal of ensuring maximum metabolite recovery with minimum disruption to the metabolome. A typical extraction protocol for recovery of polar metabolites from tissue begins with cryo-pulverization to yield a fine powder. Ice-cold extraction solvent, typically 75% 9:1 methanol:chloroform 25% water containing stable-isotope-labeled internal standards, is added to the sample to disrupt cell membranes and extract metabolites. Tissue disruption is aided by brief use of a probe sonicator followed by centrifugation and analysis of the supernatant. The Core has also participated in the development of a rapid method for the reproducible extraction of metabolites from adherent cell cultures [15].

- **Oxidative stress markers.** Oxidative stress plays a central role in several disorders including diabetes, obesity and cardiovascular disease. Dr. Pennathur's laboratory has pioneered analytical strategies to quantitatively measure amino acid oxidation markers which serve as molecular fingerprints of specific pathways of oxidation. These metabolites are quantified by isotope dilution GC/MS or ESI/LC/MS/MS and can be performed in tissues samples and biofluids as described previously [7-9, 11, 16-25].

- **Glycolytic, TCA cycle, pentose phosphate shunt metabolites.** Cellular extracts are prepared as described above and are analyzed by hydrophilic interaction liquid chromatography (HILIC) coupled to MS. The triple quadrupole is preferred for selective assays of known metabolites for which fragmentation patterns and MS parameters have been previously optimized, while TOF MS is typically used when a broader profile of the metabolome is desired, or when stable-isotope flux analysis is performed. Absolute quantitation of intracellular metabolite concentrations is generally performed by isotope dilution MS[15].

- **Amino Acid Analysis.** Purification and derivatization of biofluids or tissue extracts for amino acid analysis is performed using the "EZ:faast" kit for free amino acid analysis via GC/MS from Phenomenex [26] with norvaline added as an internal standard. The method enables quantitation of over 60 amino acids and closely related compounds.

- **NAD-related metabolome.** Nicotinamide adenine dinucleotide (NAD+) and its reduced and phosphorylated forms (NADH, NADP+ and NADPH) are important coenzymes linked to numerous cellular processes, including some that control gene expression, Ca2+ flux, cell death, and aging. An LC/MS/MS assay for quantification of 12 endogenous NAD+-related compounds, notably including nicotinamide riboside (NR) and nicotinic acid riboside (NaR) was developed [27].

- **Miscellaneous metabolite assays.** The Core has developed assays at the request of investigators. These include: purine and pyrimidines and synthesis intermediates, short chain fatty acids in plasma and in fecal samples (relating to studies of the microbiome), mevalonic acid metabolome, folic acid and sarcosine.

- **‘Fluxomics’.** There is increasing interest in utilizing specifically labeled metabolites to assess metabolism in cultured cells and, more recently, in vivo in rodents and man. The expertise of Dr. Pennathur and Dr. Ruan in the Core has provided the opportunity to extend static measurements in their system. This has been used by several laboratories to assess the
utilization of specifically labeled $^{13}$C-glucose in $\beta$-cells and in whole islets to assess pulsatile metabolism and insulin release and the effects of glucose and fatty acid exposure to alter insulin release. Additional studies have examined the alterations in glucose metabolism in the kidney in vivo and in vitro and in isolated mitochondria in diabetes and, in separate studies, skeletal muscle, liver and brain in a rat model of insulin resistance. The methodological development of these studies has required close collaboration with Dr. Nathan Qi in the Animal Phenotyping Core.

Standard Assays Offered by the Non-Directed Metabolomics Laboratory.

Three platforms have been developed for the non-targeted metabolomics analysis: reverse phase and HILIC based LC/MS run both in the ESI(+) and ESI(-) and by GC/MS. Hamilton Starlet (robotic liquid handler) is used extensively to improve consistency and reduce error rate, time, and effort.

- **Sample preparation.** For 50 $\mu$L plasma samples, Methanol/ Acetonitrile/ Acetone (MAA) Solvent (containing $^{13}$C-amino acid internal standards) is added 1:1 for protein precipitation in 96 well plates followed by centrifugation at 4 °C. Samples from cultured cells are extracted with 75% 9:1 methanol:chloroform 25% water described previously. The supernatant is directly used for HILIC-based profiling or dried under 100% N$_2$ and resuspended in water containing injection standards tertiary butyloxycarbonyl (t-BOC) aminoacids for reverse-phase separation.

- **HILIC and reverse phase chromatography/MS.** Samples are subjected to chromatography utilizing Agilent 1200 HPLC on a Phenomenex Luna NH$_2$ column (HILIC) or a Waters C18 column (reverse phase) The eluent is analyzed either in positive or negative ESI by an Agilent 6530 QTOF mass spectrometer which is coupled to the HPLC as described previously.[3, 28-30]

- **GC/MS:** Samples destined for GC/MS are dried under vacuum for a minimum of 4 hours prior to derivatization using bistrimethyl-silyl-trifluoroacetamide (BSTFA). All procedures are performed under nitrogen in a dry box. Samples are analyzed using EI on an Agilent 5975C fitted with PAL injector and equipped with HP-5MS column. Samples are injected at 5:1 split ratio and separated at 1 ml/min flow using nitrogen as carrier gas. Temperature gradient is as follows: injection at 65°C, 50°C/min to 180°C for 0.7 min, 25°C/min to 325°C for 10 min.

- **Quality Control.** Every sample set incorporates a number of pooled plasma samples and process blanks (no biological material included) prepared and analyzed simultaneously and identically with the rest of the set for QA/QC purposes. Recovery standards (10 all-$^{13}$C-amino acid), injection standards (platform specific, 10 for GC and 3 for LC), and derivatization standard (butylated hydroxyl toulene, relevant only for GC), are used to monitor sample preparation and analysis processes. As a rule, injection order is randomized using built-in LIMS function.

- **Informatics:** The Informatics system consists of three major components: the LIMS, the Data Extraction Systems (based on Agilent MassHunter platform and controlled by LIMS), and a collection of Information Interpretation and Visualization tools integrated with MetLIMS as the MetRDR interface. MetRDR tools serve library support, data curation, in-depth analysis and report generation.
• **Statistical analysis**: Metabolomics experiments generate significant amounts of data for both named compounds and "Known Unknowns". Datasets produced pose challenges similar to microarray data (large number of variables vs small number of samples). Additional dimension of complexity is introduced in big experiments due to day-to-day variations in equipment performance. Basic and advanced statistical techniques, such as the P-test (basic significance), Q-test (false discovery rate calculations), SVD (Singular value decompositions), NNMF (Non-Negative Matrix Factorizations), PCA (Principle Component Analysis), DA (Discriminant Analysis), HC (Hierarchical Clustering), SVM (Support Vector Machines), RF (Random Forest), partitioning and similarity analyses will all be applied to these datasets [31, 32]. Some of these procedures are already implemented as part of MetRDR package.

**Resources and Instrumentation**

The University of Michigan has invested about $3.5 million in developing the metabolomics infrastructure available for MDRC researchers. The resources of the Metabolomics Core are detailed in the resources page. Presently, instrumentation for Lipomics and Directed Metabolomics reside at the Brehm Center and occupies approximately 1200 sq. ft. The Non-Directed laboratory is presently located at the Traverwood facility and occupies approximately 2000 sq. ft. of space. Our current equipment inventory includes: a) Two Agilent 6890 gas chromatograph (GC) equipped with HP5973 mass detector GC/MS system with electron impact ionization (EI) and GC/ECNI/MS and positive/negative ion capabilities; b) Two Agilent Triple Quadrupole mass spectrometers (6410 and 6490) coupled with Agilent 1290 UHPLC; c) An Agilent LC/MSD Time of flight mass spectrometer which has a high mass accuracy and is ideal for directed and undirected metabolomic profiling; d) Three Quadrupole TOF instrument (Q-TOF) instruments coupled Agilent Rapid Resolution HPLC or UPLC which provide high sensitivity, high mass accuracy, high mass range, and rapid throughput ideal for metabolomic profiling; e) Additionally, the Core has an Agilent 6890 GC and a Jasco HPLC capable of analyzing a wide variety of small molecules, preparing internal standards for GC/MS and LC/MS analysis and purifying the samples prior to injection into the mass spectrometer.