**GC-TOF-MS Metabolomics Methods for Breast Tissue Processing**

Individually weighed breast tissue samples were provided frozen in tubes containing ceramic homogenization beads. Aliquots of 50-120 mg of breast tissue were mixed with degassed 3:3:2 Acetonitrile:Isopropanol:Water solution at a concentration of 100 mg/mL. Samples were homogenized in a MagNA Lyser Bead Homogenizer (Roche) for two pulses at 6500 rpm for 20 sec and place samples in cooling station (chilled at -20°C) for one minute in between the pulses. Homogenates transferred into new labeled tubes. Samples were centrifuged again at 4°C for 5 minutes at 12000 rcf and a volume of the homogenate corresponding to 50 mg breast tissue was transferred to a new set of tubes for further processing. Additionally, 100-250 µL of the doubly centrifuged homogenates were transferred to larger tubes based on phenotypes (Reduction, DCIS, HER2+, Luminal A, Luminal B, and Basal) to create phenotypic pool sample by combining equal volume aliquots from each sample in the phenotype. Equal volumes of the phenotypic pooled samples were then combined in another larger tube to create a pooled QC sample for the entire study (Total Pool 1). Pooled samples were aliquotted into analytical pool sample tubes and handled the same as the experimental sample tubes containing volumes corresponding to 50 mg of each sample. The samples were taken to dryness on the vacuum centrifuge and mixed with a 50:50 solution of Acetonitrile:Water. Samples were vortexed and centrifuged for 5 minutes at 4°C. The supernatant was removed and placed into a new tube. Samples were completely dried by vacuum centrifuge and were reconstituted by adding 630 µL of D2O (Aldrich) and 70 µL of Chenomx ISTD solution plus Imidazole as the chemical shift reference (Chenomx, Edmonton, Alberta, Canada). The samples were vortexed and centrifuged at 12000 rcf for 2 minutes. 600 µL of sample was transferred into 5mm NMR tubes and analyzed by NMR.

250 µL of the returned NMR sample was transferred to a new microcentrifuge tubes and dried by vacuum centrifuge. The samples were mixed with 1:1 Methanol:water, vortexed and taken to dryness. Dried samples were spiked with 10 µL of Methoxyamine Hydrochloride (MeOx) in pyridine (40 mg/mL) and placed onto a Thermomixer for 90 minutes at 30°C at maximum speed. After cooling to room temperature, samples were spiked with 91 µL of MSTFA+FAME mix (1 mL N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) + 10 µL fatty acid methyl ester (FAME) retention index marker solution; FAME mix contains C8, C9, C10, C12, C14, C16, C18, C20, C22, C24, C26, C28, and C30 fatty acid methyl esters) and placed onto a Thermomixer for 45 minutes at 70°C at maximum speed. Samples were cooled to room temperature before transferring to GC vials with inserts. Sample preparation procedures1 were similar to those published by Dr. Oliver Fiehn.

Samples were analyzed in 4 batches that were randomized to ensure that phenotypes were not grouped and to ensure that the holding time on the GC was random. QC pooled samples were interspersed at a regular interval during the analytical sequence. Samples were analyzed on an Agilent 7890 GC and a Leco Pegasus 4D TOF-MS. Compounds were separated and characterized on a Restek Rxi-5Sil MS capillary column (30 m X .25 mm X .25 µm with an additional 10m integrated guard column) under the instrument acquisition parameters located in Table 1. Acquisition parameters were similar to parameters established by Dr. Oliver Fiehn1.

Table 1. Instrument Acquisition Parameters

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| Carrier Gas | Helium |
| Front Inlet Mode | Splitless |
| Front Inlet Temperature | 250°C |
| Front Inlet Purge Time | 25sec after injection |
| Front Inlet Purge Flow | 40 mL/min |
| Injection Volume | 0.5 µL |
| Column Flow Rate | 1 mL/min constant flow |
| Oven Temp Initial | 50°C hold for 0.5 min |
| Oven Temp Ramp | 20°C to 330°C (@20°C/minute), hold for 5 min |
| Transfer Line Temp | 280°C |
| Source Temp | 250°C |
| Scan Range | 50 – 800 m/z |
| Scanning Cycle | 20 spectra/sec |

Following data acquisition, data files were processed for deconvolution by Leco’s ChromatTOF software and transferred to BinBase for spectral identification, peak retention index calculations and generation of a table of peak identifications and intensities.

References:

1. O Fiehn, G. Wohlgemuth, M Scholz, T Kind, DY Lee, Y Lu, S Moon and B Nikolau: Quality control for plant metabolomics: reporting MSI-compliant studies. The Plant Journal *2008;* 53:691-704.