Analysis of Fatty Acids in HBRN Human Serum Sample Cohort in collaboration with Keyur Patel, Toronto General Hospital

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Objective: Measure the levels of fatty acids in a set of 60 human serum samples.

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Short Summary:

- All samples were prepared and analyzed in the order in which they were received.
- 10 uL of a mix of 7 SIL internal standards (IS solution) was added to a plate and dried.
- 20 uL of serum was pipetted into the plate and dried.
- Samples were eluted in 100 uL MeOH, plate was centrifuged and the extract was collected in a plate underneath.
- 30 uL of water was added to the MeOH extract, and 10 uL was injected for each sample run.
- Analytes were measured semi-quantitatively by a UPLC-MS/MS negative ion method using a simple ratio against a known amount of stable-isotope internal standard.
- Quantitative values were reported in molar concentrations.

Sample Preparation

Samples were prepared using a filter-paper assisted extraction method. 6.4 mm punches of GB003 filter paper (Whatman) were inserted into wells of a 96-well Multiscreen Solvinert (Millipore) plate, after which the paper was used as a solid support for the extraction. First, 10 µL of the internal standard solution (**Table 1** below) was spotted on the paper in each well of the extraction plate with the exception of the double blank, and dried under a gentle stream of nitrogen for 5 min at room temperature (SPE Dry 96-well plate drier). Next, 20 ul of each sample including PBS for the double blank and IS-only blank, study samples, and global reference samples (NIST SRM-1950 plasma and Golden West serum) were spotted on the paper in appropriate wells, and the plate was again dried under a gentle stream of nitrogen for 20 minutes at room temperature. After drying, 100 uL of methanol was added to each well, the plate was capped, and subsequently allowed to shake at 600 rpm for 20 min at room temperature (Eppendorf Thermomixer) to extract fatty acids and internal standards from the paper. The plate was then centrifuged at 1000 rpm for 2 minutes to elute into a 96-well 1 mL collection plate (NUNC), and MeOH extracts were diluted with 30 uL water for UPLC analysis.

Compound	Delivery Solvent	MW (g/mol)	[c] of stocks in (mM)	Vol of stock used for UNC sample set (uL)	Final Vol after adding 686 uL EtOH (uL)	Final [c] in IS solution (mM)	Final [c] in samples (mM)
d8-FA 20:4 (AA)	1 mg in 100 uL methyl acetate	312.2	32.03	151.2 of 100x dilution of stock in EtOH	968.42	0.05	0.025
d5-FA 20:5 omega-3 (EPA)	1 mg in 2 mL ethanol	307.2	1.63	29.74	968.42	0.05	0.025
d5-FA 22:6 omega-3 (DHA)	1 mg in 2 mL ethanol	333.2	1.50	32.25	968.42	0.05	0.025
d4-FA 18:2 omega-6 (LA)	1 mg in 100 uL methyl acetate	284.2	35.19	13.77	968.42	0.50	0.250
d2 16:0 (palmitic acid)	dry (weighed out 25 mg in 1.5 mL ethanol	258.2	64.56	15.00	968.42	1.00	0.500
d14 FA 16:1 (palmitoleic acid)	1 mg in 1 mL ethanol	268.2	3.73	25.97	968.42	0.10	0.050
d17 FA 18:1 (oleic)	1 mg in 100 uL methyl acetate	299.2	33.42	14.49	968.42	0.50	0.250

Table 1. Concentration of each internal standard purchased as delivered from Cayman Chemical and the volumes used to make the IS solution added to each sample (10 uL of IS to 20 uL of sample).

A pool of all 60 samples was created by pipetting out an equal volume from each sample, and taking the pooled sample through the same extraction procedure as all of the study samples. This sample was injected once before, once during, and once after the study samples in order to measure the technical imprecision (measured as %CV) of the assay during the analysis of the sample cohort.

Fatty Acid Quantification by LC-MS/MS

The samples were analyzed using Ultraperformance Liquid Chromatography /Electrospray Ionization Mass Spectrometry (UPLC/ESI/MS/MS). 10 uL injections were used and UPLC separation of the lipids was performed on a Waters Acquity UPLC (Milford, MA) using a Waters Acquity 2.1 mm x 10 mm 1.7 µm CSH C18 column. Mobile phase A was 10 mM ammonium formate and 0.1% formic acid in 40/60 v/v water/MeCN. Mobile phase B was 0.1% formic acid in 10/90 v/v MeCN/2-propanol. Injections were performed at a starting condition of 0.6 mL/min and 40% mobile phase B. Gradient continued with the same flow rate and linear steps to the following conditions: 43% at 1.3 minutes, 50% at 1.4 min, 52% at 4.7 min, 99% at 5 min, 99% at 5.2 min, 40% at 5.3 min, and hold at 40% to reequilibrate for a total method time of 7 minutes.

Flow from the LC separation was introduced via negative mode electrospray ionization (ESI-) into a Q-Exactive Plus mass spectrometer (Thermo). Electrospray voltage was 3.2 kV negative, capillary temperature was 320C, a sheath gas flow of 8 and Aux gas of 1, and a probe heater temperature of 35C. Data was collected in data-dependent acquisition mode with 140,000 resolution MS1 scan from 100 to 1000 m/z, AGC target of 1e6 and 120 msec maximum IT; MS/MS scans were performed with 17,500 resolution, 5e4 AGC target, 60 msec maxiumum ID, loop count =1, 1.0 m/z isolation window, and 27V NCE. The data were imported into Skyline (https://skyline.gs.washington.edu/) for peak integration and quantification. An example of the separation of the Fatty Acids, as visualized in Skyline, is shown in **Figure 1**. Chromatographic separation along with accurate mass extraction is used to independently measure each fatty acid and internal standard.

Quantitative data analysis is performed within Skyline. A Normalized Peak Area is calculated for each fatty acid by ratioing the peak area of each fatty acid analyte to the appropriate internal standard. To calibrate the unknown values, the NIST SRM-1950 sample is defined as a Standard, since reference material has been characterized by NIST and reference values are defined for the fatty acids of interest. The NIST SRM-1950 is extracted and analyzed in triplicate on the plate, forming the basis for a single-point calibrator with slope = 1 and intercept = 0, based on the reported values of fatty acids in the NIST SRM-1950 reference material (https://www-s.nist.gov/srmors/certificates/1950.pdf). The limit of detection (LOD) was defined as 3 times the quantitative value of the blank(s). Since the NIST SRM-1950 is extracted in triplicate, the imprecision within the plate for each analyte is calculated using these samples as %CV (relative standard deviation). The triplicate measurement of the Study Pool QC (SPQC) is defined as a Quality Control sample, because the value for this sample should come out to the numerical average of the remaining unknown samples in the study. Quantitative bias is calculated as the percent difference between the average of all the samples and the SPQC. An example of such a single-point calibration, including blanks, standards, Quality control, and unknowns, is shown for FA 18:2 omega-6 (LA) in Figure 2. The Skyline file containing all raw data and MRM transitions has been uploaded to the Express repository (***4745_FA_091517.sky.zip***).

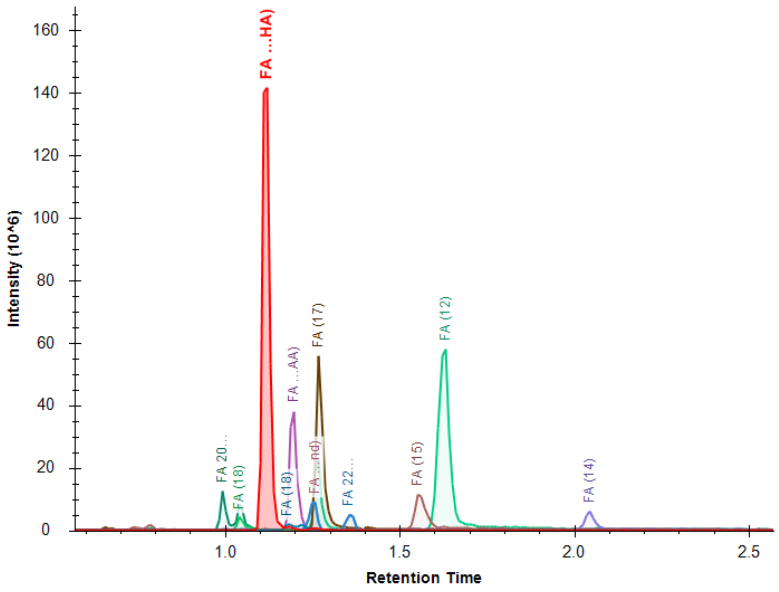

Figure 1. Extracted Ion Chromatogram of the analytes measured in the Fatty Acid panel, as visualized in Skyline.

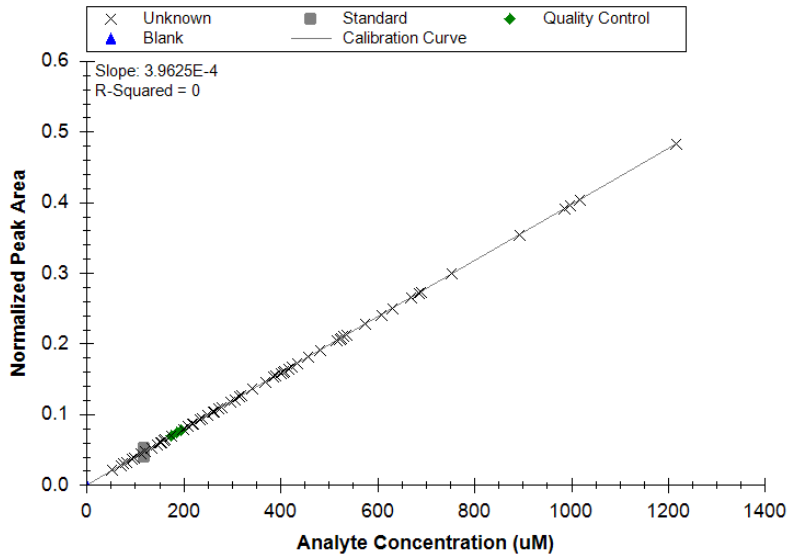

Figure 2. Example of single point calibration of Linoleic Acid using the NIST SRM-1950 plasma sample as the standard for calibration, assuming Slope =1 and intercept = 0. Unknown samples are shown as Xs, while green marks represent quality control (GoldenWest Serum Standard) and grey squares represent the NIST standard used for calibration.

Data Export and Curation

Data export and curation has been reported in the document ***4745_results_092817.xlsx*.** The run order and corresponding Sample ID to Proteomics ID numbers can be found in **Table S1** (Run Order). Based on peak integration of the analytes and internal standards (peak area), this raw data was exported from the Skyline document (4745_FA_091517.sky.zip) directly into flat file format (excel) and is reported as **Table S2** (Skyline_Export). Data was further curated in excel, to determine which analytes should be dropped because of high numbers of samples with values <LOD, or unacceptable precision or bias; these calculations are shown in **Table S3**.

(Workup). **Table S4** (Final_uM) includes the final quantitative data in micromolar (μM), excluding two analytes: FA 16:1 (palmitoleic) was recommended for exclusion because of high background in the blank (which resulted in all values being below the LOD (>3x blank) and FA 20:5 omega-6 (EPA) was excluded because of high %CV in preparation replicates (based on GoldenWest QC). ***It is recommended that Table S4 form the basis of any subsequent statistical analysis of this data.*** In Table S4, values for any analytes calculated as less than the lower limit of detection (LOD) were reported as '<LOD'. The LOD values are reported at the top of Table S4 for every analyte, and it is recommended that any missing values be replaced by the LOD for subsequent statistical analysis. Finally, **Table S5** (LODreplaced_log2) contains the data with missing values replaced with LOD, and the data log2 scaled. This represents one potential pretreatment of the data, followings standard procedure for missing value replacement and log2 scaling (to give

Data Quality Control

Table S5 data was used to perform a principal components anlysis (PCA) to look for outlier samples, using REML estimation method in JMP Pro v13 (SAS Institute, Cary, NC). Figure 3 shows the results of this PCA. As expected, the SPQC (study pool, red) samples group tightly together and in the middle of all other study samples. There were no outlier samples detected.

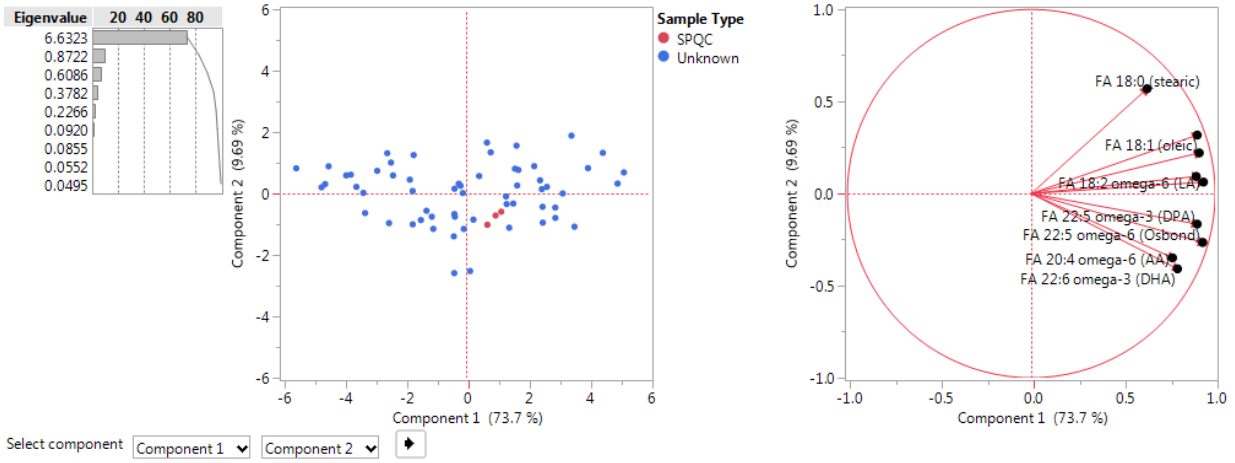

Figure 3. Principal Components analysis of fatty acids data from Table S5, log2 transfomed data with REML.

Additionally, a 2D Hierarchical clustering analysis was performed on standardized log2 data (Table S5), shown in Figure 4, also using JMP Pro v13. Since we are blinded to the study groups, we can't say whether the groups are clustering as expected, but as expected, we see study pool samples cluster together, as well as most samples having either high or low values of free fatty acids, collectively. Stearic acid (FA 18:0) shows a SPQC value below the mean (dark blue in the cluster), indicating positive bias in some of the quantitative values (overestimation of some samples at or near the LOD. This is not unexpected, as 22% of the samples were <LOD for Stearic acid (FA 18:0), Table S3. Otherwise, the SPQC values are near the mean for all analytes.

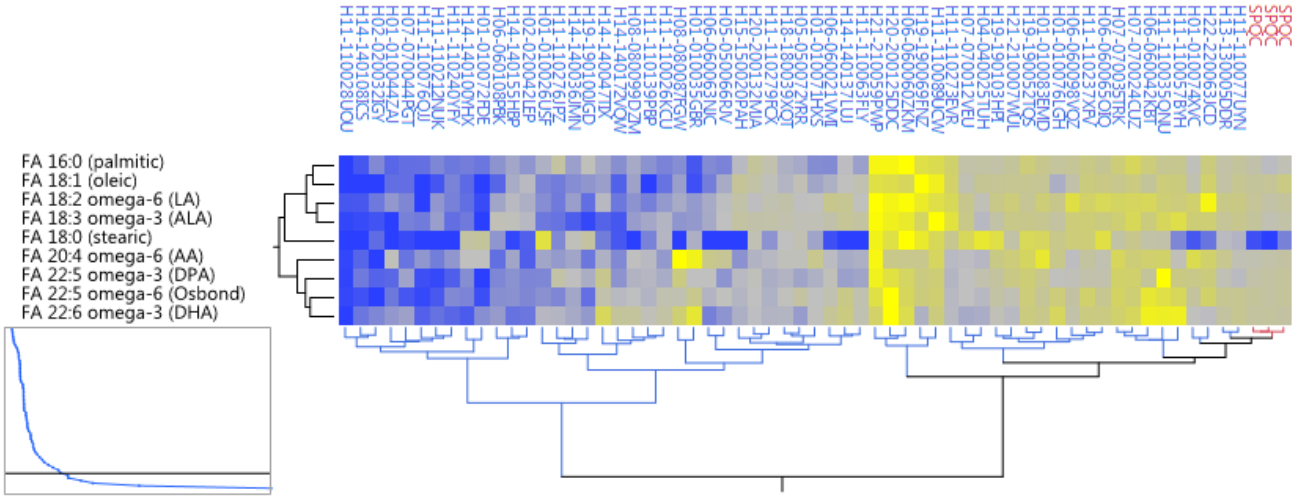

Figure 4. 2D Hierarchical Clustering of log2 fatty acids data Table S5, using robust standardization (i.e. Z-Score transformation) in JMP Pro 13. Yellow indicates a high value, (+2 standard deviations from the mean) while dark blue means a low value (-2 standard deviations from the mean).