Michels\_CHEAR\_2017\_1977

Urine Metabolomics

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Enclosed Files:

“Meta\_2019\_Michels2017\_1977.csv”

“Michels\_Urine\_ZHP\_targeted\_Final.csv”

“Michels\_Urine\_RPN\_targeted\_Final.csv”

“Michels\_Urine\_RPN\_Untargeted\_Final.csv”

“Michels\_Urine\_ZHP\_Untargeted\_Final.csv”

Objective

To perform an untargeted metabolomics analysis of urine samples, matrix blanks and quality control samples. The metabolomics approach will be performed using both reverse phase (RP) and HILIC chromatography (ZHP) separations coupled to high-resolution mass spectrometry.

Samples

Samples were received and stored at -80°C until processing. In total, 315 samples had sufficient sample volume for metabolomics analysis.

Experimental

Solutions and solvents: All solutions are prepared at room temperature using LC-MS grade solvents and analytical grade reagents. Acetonitrile and formic acid (Optima LC/MS grade) were purchased from Fisher Scientific. Isopropanol (hypergrade for LC-MS LiChrosolv) was purchased from Sigma. Ultrapure water was filtered with a Millipore Milli-Q system (18mU).

Sample preparation:

Aliquoting was initially performed for all CHEAR assays, and included an aliquot of each sample for metabolomics and specific gravity measurements. Specific gravity measurements were made first, to determine dilution factors required for pre-acquisition normalization. Specific gravity and dilution factors are included in the “Meta\_2019\_Michels2017\_1977.csv”

Urine samples were thawed on ice in batches of approximately 65 samples, and vortexed. The sample was diluted with water down to a specific gravity of 1.002 for pre-acquisition normalization. A 20 L aliquot of the diluted sample was prepared for metabolomics analysis. In addition, a 20 L aliquot from each diluted sample was combined for use as a pooled quality control sample and re-aliquoted into 20L samples. Samples were then returned to -80°C until analysis.

Extraction was performed in batches of approximately 65 samples, immediately prior to LC-HRMS analysis. All samples in a batch were thawed on ice, combined with 180L of acetonitrile containing internal standards, and vortexed for 30sec. Samples were then centrifuged (13000 g, 15 min, °C), and 60 L of supernatant transferred to two LC vials for RP and HILIC analysis. Extract remainder was returned to -80°C. Following the same protocol 20 L aliquots each of a matrix blank (replacing the urine with H2O, “matrix”), a CHEAR Reference urine sample (global quality control, “UT”), a NIST 3672 sample (global quality control, “NIST”), and multiple pooledQC samples (local quality control, “LQC”) were extracted.

LC-HRMS analysis: Sample extracts were analyzed in ZIC HILIC positive (ZHP) and RP negative (RPN) modes separately using an ultra-high performance liquid chromatography (UHPLC) 1290 Infinity II system (including 0.3 µm inline filter, Agilent Technologies, Santa Clara, USA) with 1260 Infinity II isocratic pump (including 1:100 splitter) coupled to a 6550 iFunnel or 6545 quadrupole-time time of flight (Q-TOF) mass spectrometer with a dual AJS electrospray ionization source (Agilent Technologies, Santa Clara, USA). Samples were maintained at 5°C in the autosampler module. Reference masses included positive ionization mode: purine ([M+H]+ *m/z* 121.0509), HP-0921 ([M+H]+ *m/z* 922.0098); and negative ionization mode: purine ([M-H]- *m/z* 119.0363), HP-0921 ([M+COOH]- *m/z* 966.0007). Sheath and drying gas (Nitrogen purity >99.999%) flows were 8 L/min and 17 L/min, respectively. Drying and sheath gas was 250 °C, with the nebulizer pressure at 35 psig, and voltages for positive and negative ionization modes at +3000 V and -3000 V, respectively.

*ZHP analysis.* For polar metabolites separation, 2 L of sample was injected onto a HILIC SeQuant® ZIC®-HILIC column (100 mm × 2.1 mm, 100 Å, 3.5 µm particle size, Merck, Darmstadt, Germany) maintained at 25°C. Separation occurred using Mobile phase A consisted of water with 0.1% formic acid and Mobile phase B consisted of Acetonitrile with 0.1% formic acid at a flow rate of 0.3 mL/min as described in Table 1. Data was acquired with a mass range of 40-1200 *m/z*

Table 1. HILIC separation

|  |  |  |
| --- | --- | --- |
| **Time [min]** | **Solvent A %** | **Solvent B %** |
| 0 | 5 | 95 |
| 1.5 | 5 | 95 |
| 12 | 60 | 40 |
| 14 | 60 | 40 |
| 14.2 | 75 | 25 |
| 17 | 75 | 25 |
| 18 | 5 | 95 |
| 25 | 5 | 95 |

*RPN analysis:* For nonpolar metabolites separation, 2 L of sample sandwiched between 10 L of water was injected onto a Zorbax Eclipse Plus C18, RRHD column (50 mm × 2.1 mm, 1.8 µm particle size, Agilent Technologies, Santa Clara, USA) coupled to a guard column (5 mm × 2 mm, 1.8 µm Agilent Technologies, Santa Clara, USA) maintained at 50°C. Separation occurred using Mobile phase A consisted of water with 0.1% formic acid and Mobile phase B consisted of 2-propanol:ACN (90:10, *v/v*) with 0.1% formic acid at a flow rate of 0.4 mL/min as described in Table 2. Data was acquired with a mass range of 50-1200 *m/z*

**Table 2.** Gradient settings for RP chromatography

|  |  |  |
| --- | --- | --- |
| **Time [min]** | **Solvent A %** | **Solvent B %** |
| 0 | 95 | 5 |
| 2 | 95 | 5 |
| 4 | 70 | 30 |
| 13.5 | 2 | 98 |
| 15 | 2 | 98 |
| 15.5 | 95 | 5 |
| 19 | 95 | 5 |

The HRMS instrument was tuned prior to every batch. The samples were analyzed in 5 batches per mode. Column was conditioned with data-dependent injections (DDA), and a series of QCs and blanks were run followed by the samples. LQC and UT samples were injected throughout the run. MSMS data was obtained during column conditioning (DDA) and at the end of each batch (DDA, DIA) to facilitate annotation.

Data Processing:

Analysis was performed for all batches (5) in each mode.

*Database dependent targeted identification:* Metabolites were identified based upon in-house database matching considering retention time, accurate mass, and MSMS matching (when available) matching with pure standards analyzed under the same conditions.

*Untargeted metabolomics analysis*: Parameters for peak picking grouping, and alignment with ‘XCMS’ included centwave feature detection, orbiwarp retention time correction, minimum fraction of samples in one group to be a valid group = 0.25, isotopic ppm error = 10. Width of overlapping *m/z* slices (mzwid) = 0.003 or 0.015, and retention time window (bw) = 12.4 s and 22 s for ZHP and RPN, respectively. Minimum and maximum peak width were 5 and 20 s for reverse phase and 10 and 60 s for HILIC. The resulting peak table of retention times, *m/z* values, and peak areas was exported for data processing.

Annotation of the untargeted data was facilitated by xMSannotator using the annotation scheme of Schymanski et al. (Environmental Science & Technology, 2014). Level 1 and 2 annotations were those that were confirmed with database dependent annotation. Lower confidence annotations (level 4) are those from the HMDB and T3DB online databases that were highly ranking by xMSannotator. Level 5 annotations were named by “mz\_rt”.

Metadata for the analysis including the batch and run order of each injection are provided in the Metafile, “Meta\_2019\_Michels2017\_1977.csv”. We also included the Specific gravity measurements and dilution factor performed for each sample prior to data acquisition.

Results

Our in-house library contains information for 301 metabolites (ZHP = 205 and RPN = 96) representing biochemically and environmentally important compounds. Metabolite identification was based on matching accurate mass/retention time (AMRT) in combination with tandem mass spectrometry when available by DDA and the all ion fragmentation (AIF) mode. Identified/annotated metabolites are reported in the files. Identified metabolites from the database dependent matching are reported in the csv files ‘’Michels\_Urine\_ZHP\_targeted\_Final.csv”, and “Michels\_Urine\_RPN\_targeted\_Final.csv”. Cells with zero indicate peaks that were below the limit of detection. Identifications with “/” indicate that analytical standards co-eluted and cannot be distinguished by AMRT matching. Untargeted results are reported in the csv files, “Michels\_ZHP\_Untargeted\_Final.csv”, and “Michels\_RPN\_ Untargeted\_Final.csv. Cells with zeros indicate missing peaks.

**Note:** All urine samples were pre-diluted to a specific gravity of 1.002 prior to data acquisition. Therefore, no further adjustments for urinary dilution factor are needed during data analysis.