# Quantitative measurement of gut microbial metabolites in human serum samples ADNI1, 2 and GO cohorts

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## Summary

The purpose of this project was to measure a panel of representative gut microbial metabolites including bile acids in human serum samples.

Biospecimens of human serum (ADNI samples) were provided by Duke University (the sponsor), and the quantitative measurement of these samples was carried out using both ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS).

The major findings are summarized below:

- We have quantitated a total of **104 metabolites** including 33 bile acids in human serum samples. The results are provided in separate excel file attached to the report.

## Materials and Methods

### *Samples*

Human serum samples were provided by Dr. Kaddurah-Daouk from Duke University (the sponsor) and received on January 29, 2019 for ADNI GO/2 project and August 18, 2020 for ADNI **1** project. After sample check-in, all the samples were immediately stored at -80°C (Forma 8600 series ultra-low temperature freezer, Thermo-Fisher Scientific, Nashville, NC) until sample preparation and analysis.

### *Chemicals*

All of the 57 bile acid standards were obtained from Steraloids Inc. (Newport, RI) and TRC Chemicals (Toronto, ON, Canada), and 9 stable isotope- labeled standards were obtained

from C/D/N Isotopes Inc. (Quebec, Canada) and Steraloids Inc. (Newport, RI). The standards and stable isotope- labeled standards were accurately weighed and prepared in methanol at a concentration of 5.0 mM (stock solution). Further dilution was performed to obtain a series of calibration concentration of 2000, 400, 160, 32, 12.8, 2.5, or 1 nM with methanol/water (50/50, v/v). Internal Standard (IS) concentrations were kept constant at all the calibration points at 100 nM for GCA-d4, TCA-d4, TCDCA-d9, UDCA-d4, CA-d4, GCDCA-d4, GDCA-d4, DCA-d4, and 200 nM for LCA-d4.

All standard compounds of other gut microbial metabolites and stable isotope-labeled internal standards were commercially purchased from Sigma-Aldrich (St. Louis, MO), Nu-Chek Prep (Elysian, MN), Steraloids Inc. (Newport, RI), and TRC Chemicals (Toronto, ON, Canada). The stock solutions of all standards were prepared in HPLC grade methanol or ultrapure water with a concentration of either 5 or 1 mg $mL^{-1}$.

Methanol (Optima LC-MS), acetonitrile (Optima LC-MS) and formic acid (Optima LC-MS) were purchased from Thermo-Fisher Scientific (FairLawn, NJ). Ultrapure water was produced by a Mill-Q Reference system equipped with a LC-MS Pak filter (Millipore, Billerica, MA).

The derivatization regents, 3-nitrophenylhydrazine (3-NPH) and N-(3-(dimethylamino)propyl)-N'- ethylcarbodiimide (EDC)-HCl were purchased from Sigma-Aldrich (St. Louis, MO).

## *Sample Preparation for bile acid analysis by UPLC-TQMS[1-2]*

The bile acid-free matrix (BAFM) was used to prepare bile acid calibrators in order to minimize analytical variations and compensate matrix effects. The BAFM was obtained using the following charcoal-stripping protocol. A total of 3-g activated charcoal was added to 20 mL of plasma (Sigma-Aldrich, St. Louis, M.O.), respectively. The mixture was shaken at 25°C overnight, and the supernatant was obtained by ultra-centrifugation. The supernatant was further filtered with 0.22-µm membrane filter and evaluated for the presence of bile acids before use. The calibrators were prepared in the blank matrix and processed as the same way as was used for the extraction of bile acids from the biological samples, such that the analytical variations can be monitored and the matrix effects are compensated.

At the time of analysis, samples were thawed on ice-bath to diminish sample degradation. The sample preparation followed a published method with modifications[1 2] Briefly, each 50 µL of serum, or standard solution was spiked with 150 µL of acetonitrile containing 9 internal standards and the extraction of bile acids was conducted at a laboratory shaker at 10°C and 1,500 rpm for 20 min. After centrifugation, the supernatant was transferred to a micro-centrifuge tube for lyophilization using a FreeZone freeze dryer system (Labconco, Kansas City, MO). The residue was reconstituted with 50 µL of mobile phase B (acetonitrile / methanol =95:5, v/v) and 50 µL of mobile phase A (water with formic acid, pH=3.25), and centrifuged at 13,

500 g and 4 °C for 20 min. The supernatant was transferred to a 96-well plate for LC-MS analysis and the injection volume was 5 µL.

***Sample Preparation for other gut microbial metabolite analysis by UPLC-TQMS³***

The sample preparation following a published method with modifications. Briefly, an aliquot of 20 µL of each standard solution or serum samples were mixed with 120 µL internal standards solution. After centrifugation at 13 500 g and 4 °C for 10 min, an aliquot of 30 µL of the supernatant was carefully transferred to a 96 well plate for derivatization. A 10 µL aliquot of freshly prepared derivative reagents (200 mM 3-NPH in 75% aqueous methanol and 96 mM EDC-6% pyridine solution in methanol) was added to each well. The plate was sealed and the derivatization was carried out at 30 °C for 60 min. After derivatization, 400 µL of ice-cold 50% methanol solution was added to dilute the sample. Then the plate was stored at -20 °C for 20 min and followed by 4000g centrifugation at 4 °C for 30 min. 135 µL of supernatant was transferred to a new 96-well plate in each well. Finally, the plate was sealed for LC-MS analysis and the injection volume was 5 µL.

A Waters ACQUITY ultra performance LC system coupled with a Waters XEVO TQ-S mass spectrometer with an ESI source controlled by MassLynx 4.1 software (Waters, Milford, MA) was used for all analyses. Chromatographic separations were performed with an ACQUITY BEH C18 column (1.7 µm, 100 mm x 2.1 mm internal dimensions) (Waters, Milford, MA). UPLC-MS raw data obtained with negative mode were analyzed using TargetLynx applications manager version 4.1 (Waters Corp., Milford, MA) to obtain calibration equations and the quantitative concentration of each bile acid in the samples.

## Instrumentation

An ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) system (ACQUITY UPLC-Xevo TQ-S, Waters Corp., Milford, MA) was used to quantitate bile acids in human serum samples. The optimized instrument settings are briefly described in Table 1 and Table 2.

| **Table 1. UPLC-MS/MS instrument settings** | |
|---|---|
| **UPLC** | |
| **column** | ACQUITYUPLC BEH Cl8 1.7 µM VanGuard pre-column (2.lx5 mm) andACQUITY<br>UPLC BEH Cl8 1.7 µM analytical column (2.1 x 100 mm) |
| **column temp. (°c)** | 45 |
| **sample manager temp. (°c)** | 10 |
| **mobile phases** | A=water with formic acid (pH =3.25); and B=acetonitrile / methanol (95:5) |
| **gradient conditions** | 0-1 min (5% B), 1-5 min (5-25% B), 5-15.5 min (25-40% B), 15.5-17.5 min (40-95% B),<br>17.5-19 min (95% B), 19-19.5 min (95-5% B), 19.6-21 min (5% B). |

| | |
|---|---|
| **flow rate (ml/min)** | 0.45 |
| **Mass spectrometer** | |
| **capillary (kv)** | 1.2 (ESH |
| **source temp (°c)** | 150 |
| **desolvation temp (°c)** | 550 |
| **desolvation gas flow (11hr)** | 1200 |

| **Table 2. UPLC-MS/MS instrument settings for other microbial metabolites platform** | |
|---|---|
| **UPLC** | |
| **column** | ACQUITY UPLC BEH Cl8 1.7 µM VanGuard pre-column (2.lx5 mm) and ACQUITY UPLC BEH Cl8 1.7 µM analytical column (2.1 x 100 mm) |
| **column temp. (°c)** | 40 |
| **sample manager temp. (°c)** | 10 |
| **mobile phases** | A=water with formic acid (pH =3.25); and B=acetonitrile / isopropanol (70:30) |
| **gradient conditions** | 0-1 min (5% B), 1-5 min (5-30% B), 5-9 min (30-50% B), 9-11 min (50-78%B), 11-13.5 min (78-95%B), 13.5-14 min (95-100%B), 14-16 min (100%B,0.6mL/min), 16-16.1 min (100-5% B), 16.1-18 min (5% B). |
| **flow rate (ml/min)** | 0.40/0.6 |
| **Mass spectrometer** | |
| **capillary (kv)** | 1.5 (ESI+), 2.0 (ESH |
| **source temp (°c)** | 150 |
| **desolvation temp (°c)** | 550 |
| **desolvation gas flow (11hr)** | 1000 |

### *Data Analysis*

The raw data files generated by UPLCTQMS were processed using the TMBQ software (v1.0, HMI, Shenzhen, Guangdong, China) to perform peak integration, calibration, and quantification for each metabolite.

Mass spectrometry- based quantitative metabolomics refers to the determination of the concentration of a substance in an unknown sample by comparing the unknown to a set of standard samples of known concentration (i.e., calibration curve). The calibration curve is a plot of how the analytical signal changes with the concentration of the analyte (the substance to be measured). For most analyses a plot of instrument response vs. concentration will show a linear relationship. This yields a model described by the equation y = ax + b, where y is the instrument response e.g., peak height or area, a represents the slope / sensitivity, and b is a constant that describes the background. The analyte concentration (x) of unknown samples may be calculated from this equation.

### *Sample Control Procedure (BPG010-R02)*

Each sample received was accessioned into the UHCC MSR LIMS system and was assigned by the LIMS a unique identifier, which was associated with the original source identifier only. This identifier was used to track all sample handling, tasks, results etc. The samples and aliquots were bar-coded and tracked by the LIMS system. All portions of any sample were automatically assigned their own unique identifiers by the LIMS when a new task was created; the relationship of these samples was also tracked. All samples were maintained at -80 °C until processed.

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***Data Control Procedure (BPG011-R02)***

The UHCC MSR DCP (BPG011-R02) was used to ensure the data security. The data retained on instrument control computers are immediately transferred to a local data server (UHCC MSR) located in a locked room.

***Quality Control Procedure***

In addition to the internal standards used for quality control (QC), another two types of QC samples, including test mixtures (a group of commercially available standards with a mass range across the system mass range at 3 concentrations (low, medium and high) within the range of the calibration curve), and pooled biological samples were used for our metabolomic procedures. The QC samples were prepared along with the study samples and run after each 20 serum samples. The QC samples were kept at 10 ℃ during the entire analysis.

The QC samples were evenly inserted in each set of the analysis running sequence to monitor the stability of the large-scale analysis. The noise baseline was established from reagent blank measurement and any metabolite with signal to noise ratio ≤ 3.0 was rejected from statistical analysis. Relative standard deviations (RSDs) of each metabolite in the test mixtures (low, middle, high) and pooled QC samples measured were calculated. The relative standard deviation for the lower concentration metabolites in the reference standard mixture were less than 30% and the R.S.D. for the higher concentration metabolites shall be better than 15% for each batch of sample analysis.

## Version Information 1

### Dataset Information

This methods document applies to the following dataset(s) available from the ADNI repository:

| Dataset Name | Date Submitted |
|---|---|
| ADMC U Hawaii UPLC-MS/MS Gut Metabolites Serum Longitudinal [ADNI1,GO,2] | 19/10/2021 |

## References

## About the Authors

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