SOP_Assay: Measurement of human TMAO, ADMA and SDMA by Liquid Chromatography-Tandem Mass spectrometry (LC-MS/MS)

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1. **Purpose**
This Standard Operating Procedure (SOP) describes the process for the quantitative assessment of uremic toxins, Trimethylamine N-oxide (TMAO), Asymmetric Dimethylarginine (ADMA) and Symmetric Dimethylarginine (SDMA) in human plasma/serum and urine.

2. **Introduction**
Trimethylamine N-oxide (TMAO) is the organic compound which is a product of oxidation of trimethylamine, a common metabolite in animals. TMAO associates with cardiovascular disease in the general population. TMAO accumulates to very high levels in people receiving chronic hemodialysis as measured at the peak midweek pre-dialysis (Xin Hai et al., 2015). High levels of ADMA and SDMA levels also indicate chronic kidney disease (CKD). This SOP describe assays for studies designed to evaluate the utility of kidney injury markers and inflammatory markers to predict the progression of kidney disease by measuring TMAO, ADMA and SDMA using mass spectrometry.

3. **Procedure summary**
**Background:** Plasma/serum concentrations of TMAO, ADMA and SDMA are determined using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) approach incorporating a multiple reaction-monitoring (MRM) step.

4. **Location**
Biomedical Research Building (BRB) 10th floor.
Department of Medicine/Case Western Reserve University.
Sub Division of Nephrology and Hypertension

5. **Required equipment/reagents**
   **A. Reagents and equipment:**
   1) Windows 7 professional, Microsoft office 2007
   2) Refrigerated Centrifuge (Eppendorf 5417R Rotor FA 45-30-11, max 20,000 g)
   Note- centrifuges are maintained on an as need basis and are not routinely calibrated
   3) Vortex (DAIGGER Vortex Genie 2 Cat. No. 3030A)
   Note- Vortexers are maintained on an as need basis and are not routinely calibrated
   4) Multitube Vortexer (VWR DVX-2500) - Speed of 1500 for 2 min for the vortex by turning on the power button.
   5) Zebra printer GX430t™- 300 dpi IE (item # KBSGX4 3102410000)
   6) Single channel manual pipettes
      i. Rainin Classic 0.5 µl-10µl
      ii. Rainin Classic 10 µl-100 µl
      iii. Rainin Classic 100 µl-1000 µl
   Note- pipettes are serviced every 6-months on site using an commercial provider of Calibration services.
   7) Shimadzu Prominence LC system (Kyoto, Japan) coupled to an API 4000 triple Quadrupole mass spectrometer (AB Sciex, Canada).
8) Pipette Tips
   i. 10 µl Filtered Tips- Catalog no: 1121-4810
   ii. 200 µl Filtered Tips- Catalog no: 1120-8810
   iii. 1000 µl Filtered Tips- Catalog no: 1122-1830
9) Auto sampler vials 2ml (Thermo Scientific-National Part no: C4000-1W)
10) Blue screw caps (Agilent, Part no: 5182-0717)
11) 250 µl insert polypropylene (Agilent, Part no: 5182-0849)
12) 1.5 ml microcentrifuge tubes (ependorf tubes) flat top snaplock caps liquid tight
    and reliable seal with graduated markings. (Dot Scientific inc- catalog no: 607-
    GMT)
13) Personal Protective Equipment (PPE)
14) Lab coat
15) Gloves (Kimberly-Clark purple nitrile powder free exam gloves, catalog no:
    KC500)
16) Goggles
17) HPLC grade methanol (Fisher Scientific Company LLC- catalog no: A4524)
18) HPLC grade water 4 L (Fisher Scientific Company LLC-catalog no: 91531)
19) Formic Acid MASS SPECTR 50ML (Fisher Scientific Company LLC-catalog no:
    6000617)
20) ADMA-d7 From Cambridge isotope labs (catalog number: DLM-7476-5)
21) NG, NG'-Dimethyl-L-arginine-d6 (SDMA-d6) From Toronto Research
    Chemicals (catalog number: D463582)
22) NG, NG-Dimethyl arginine dihydrochloride (ADMA) (catalog number: D4268)
23) N-G,N-G'-Dimethyl-l-arginine (SDMA) (catalog number: D0390)
24) Trimethylamine n-oxide hydrochloride (TMAO) (CAS No. 1184-78-7)
25) Trimethylamine n-oxide (TMAO-d9) (D9, 98%) (catalog no: DLM-4779-1)-
    Cambridge isotope labs

26) LC Solvents  

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile phase ‘A’</td>
<td>Water with 0.1 % Propionic acid</td>
</tr>
<tr>
<td>Mobile phase ‘B’</td>
<td>Methanol with 0.1 % acetic acid</td>
</tr>
<tr>
<td>Mobile phase ‘C’</td>
<td>50 % methanol with 0.2 % Formic acid</td>
</tr>
</tbody>
</table>

The buffers are prepared weekly, filtered (by ‘Millipore’ vacuum driven sterile filtration system-
disposable purchased from Millipore, Catalog no: SCGPU01RE) and stored at room temperature. The buffers are prepared weekly, filtered (by ‘Millipore’ vacuum driven sterile filtration system-
disposable purchased from Millipore, Catalog no: SCGPU01RE) and stored at room temperature. Mobile phase A is prepared by adding 900 mL of HPLC grade water to a 1L class A graduated cylinder and adding 1 mL of propionic acid and finally filling with HPLC water to the 1000 mL mark. Mobile phase A is then poured into a 1 L Mobile Phase bottle labeled with Water 0.1% propionic acid. Mobile phase B is prepared by adding 900 mL of HPLC grade water to a 1L class A graduated cylinder and adding 1 mL of acetic acid and finally filling with HPLC water to the 1000 mL mark. Mobile phase B is then poured into a 1 L Mobile Phase bottle labeled with Water
0.1% acetic acid. Mobile phase C is prepared by adding methanol to a 1L glass graduated cylinder and then adding 2 mL of formic acid and finally filling with HPLC water to the 1000 mL mark. Mobile phase C is then poured into a 1 L Mobile Phase bottle labeled with water, methanol and 0.2 % formic acid.

**B. Construction of Calibration curve:**

i. **Preparation TMAO, ADMA and SDMA calibrators:**

Known concentrations of TMAO, ADMA and SDMA are used for constructing the calibration curve. The concentration range of TMAO used are 0.5 µM, 1.5 µM, 7.5 µM, 15 µM, 30 µM, 75 µM, 100 µM, 150 µM and 200 µM. TMAO stock concentrations that are prepared in HPLC grade water are 1 mM, 100 µM and 20 µM (Table 2). The main stocks are prepared every 6 months. Calibration working solutions are made by using these stock concentrations (see Table 3 for the dilutions) to prepare the calibration curve. Calibration standards are prepared in a volume of 500 µl and made every month.

ADMA and SDMA concentration range used to construct calibration curve is that, 0.05 µM, 0.25 µM, 0.5 µM, 1 µM, 2 µM, 3 µM, 4 µM, 5 µM and 10 µM. ADMA and SDMA stock concentrations that are prepared in HPLC grade water are 100 µM, 10 µM and 1 µM (table 2). Calibration working solutions of ADMA and SDMA are made by using these stock concentrations (table 3) to prepare the calibration standards and thus the calibration curve. Calibration standards are prepared in a volume of 500 µl and made every month.

The working stocks are stored at -20 °C in aliquots for a month. To prepare the calibration curve, to 20 µl of each working stock, 80 µl internal standard mixture (see Table 5 for the preparation of internal standard mixture) and extracted similar to the samples by vortexing, centrifugation and collecting the supernatant into the auto sampler vial for injection into mass spectrometry (Table 4). The calibration standards are prepared on the same day along with the quality controls and unknowns of that particular batch (batch is defined as a set of samples run each day).

Main stocks and working stocks have all three compounds TMAO, ADMA and SDMA together prepared in water.

| Table 2: Main Stocks of TMAO, ADMA and SDMA (prepared every 6 months): |
|-----------------------------|-----------------------------|
| **Analyte**                | **Main Stock concentration** |
| TMAO                       | 20 µM, 100 µM and 1 mM      |
| ADMA                       | 1 µM, 10 µM and 100 µM       |
| SDMA                       | 1 µM, 10 µM and 100 µM       |
### Table 3: Preparation of Working Stocks (500 µl prepared every month):

<table>
<thead>
<tr>
<th></th>
<th>TMAO concentration (µM)</th>
<th>ADMA concentration (µM)</th>
<th>SDMA concentration (µM)</th>
<th>Volume from the TMAO stocks</th>
<th>Volume from the ADMA stocks</th>
<th>Volume from the SDMA stocks</th>
<th>Water (to make up to 500 µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Std 1</td>
<td>0.5</td>
<td>0.05</td>
<td>0.05</td>
<td>12.5 µl (from 20 µM TMAO)</td>
<td>25 µl (from 1 µM stock)</td>
<td>25 µl (from 1 µM stock)</td>
<td>437.5 µl</td>
</tr>
<tr>
<td>Std 2</td>
<td>1.5</td>
<td>0.25</td>
<td>0.25</td>
<td>37.5 µl (from 20 µM TMAO)</td>
<td>125 µl (from 10 µM stock)</td>
<td>125 µl (from 10 µM stock)</td>
<td>212.5 µl</td>
</tr>
<tr>
<td>Std 3</td>
<td>7.5</td>
<td>0.5</td>
<td>0.5</td>
<td>187.5 µl (from 20 µM TMAO)</td>
<td>25 µl (from 10 µM stock)</td>
<td>25 µl (from 10 µM stock)</td>
<td>262.5 µl</td>
</tr>
<tr>
<td>Std 4</td>
<td>15</td>
<td>1</td>
<td>1</td>
<td>375 µl (from 20 µM TMAO)</td>
<td>50 µl (from 10 µM stock)</td>
<td>50 µl (from 10 µM stock)</td>
<td>25 µl</td>
</tr>
<tr>
<td>Std 5</td>
<td>30</td>
<td>2</td>
<td>2</td>
<td>150 µl (from 100 µM TMAO)</td>
<td>100 µl (from 10 µM stock)</td>
<td>100 µl (from 10 µM stock)</td>
<td>150 µl</td>
</tr>
<tr>
<td>Std 6</td>
<td>75</td>
<td>3</td>
<td>3</td>
<td>375 µl (from 1 mM TMAO)</td>
<td>150 µl (from 10 µM stock)</td>
<td>150 µl (from 10 µM stock)</td>
<td>162.5 µl</td>
</tr>
<tr>
<td>Std 7</td>
<td>100</td>
<td>4</td>
<td>4</td>
<td>500 µl (from 1 mM TMAO)</td>
<td>200 µl (from 10 µM stock)</td>
<td>200 µl (from 10 µM stock)</td>
<td>50 µl</td>
</tr>
<tr>
<td>Std 8</td>
<td>150</td>
<td>5</td>
<td>5</td>
<td>750 µl (from 1 mM TMAO)</td>
<td>25 µl (from 100 µM stock)</td>
<td>25 µl (from 100 µM stock)</td>
<td>375 µl</td>
</tr>
<tr>
<td>Std 9</td>
<td>200</td>
<td>10</td>
<td>10</td>
<td>1000 µl (from 1 mM TMAO)</td>
<td>50 µl (from 100 µM stock)</td>
<td>50 µl (from 100 µM stock)</td>
<td>300 µl</td>
</tr>
</tbody>
</table>

### Table 4: Calibration standards preparation (prepared with each batch (a set of samples that are run on one day)):

<table>
<thead>
<tr>
<th>TMAO, ADMA AND SDMA</th>
<th>From Working stock</th>
<th>Internal std mixture in methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Std 1</td>
<td>20 µl</td>
<td>80 µl</td>
</tr>
<tr>
<td>Std 2</td>
<td>20 µl</td>
<td>80 µl</td>
</tr>
<tr>
<td>Std 3</td>
<td>20 µl</td>
<td>80 µl</td>
</tr>
<tr>
<td>Std 4</td>
<td>20 µl</td>
<td>80 µl</td>
</tr>
<tr>
<td>Std 5</td>
<td>20 µl</td>
<td>80 µl</td>
</tr>
<tr>
<td>Std 6</td>
<td>20 µl</td>
<td>80 µl</td>
</tr>
<tr>
<td>Std 7</td>
<td>20 µl</td>
<td>80 µl</td>
</tr>
<tr>
<td>Std 8</td>
<td>20 µl</td>
<td>80 µl</td>
</tr>
<tr>
<td>Std 9</td>
<td>20 µl</td>
<td>80 µl</td>
</tr>
</tbody>
</table>

### ii. Internal standards (isotope dilution method)

TMAO-d9 is the internal standard used for the TMAO calibration curve as well as for sample TMAO measured. TMAO-d9 is obtained from Cambridge Isotope Laboratories. 10 mM of TMAO-d9 stock is prepared from which 10 µM of TMAO-d9 working concentrations in methanol are prepared (Table 5 for the dilutions).

ADMA-d7 and SDMA-d6 are used as internal standards for measuring ADMA and SDMA in the samples. 1 mM of ADMA and SDMA stocks are prepared from which 1 µM of ADMA-d7
and SDMA-d6 working concentrations in methanol are used. Main stocks prepared once every 3 months.

Table 5:

<table>
<thead>
<tr>
<th>Internal standard Analyte</th>
<th>Weight/in water</th>
<th>Molecular weight</th>
<th>STOCK CONCENTRATION (in Water)</th>
<th>Working stocks (in Water)</th>
<th>Amount taken (make up to 50 ml with Methanol) to make the mixture WORKING CONCENTRATION (in Methanol) prepared with every batch</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMAO-d9</td>
<td>8.417 mg/10ml</td>
<td>84.17</td>
<td>10 mM</td>
<td>10 µM</td>
<td>50 µl</td>
</tr>
<tr>
<td>ADMA-d7</td>
<td>2.6377mg/10ml</td>
<td>263.77</td>
<td>1 mM</td>
<td>1 µM</td>
<td>50 µl</td>
</tr>
<tr>
<td>SDMA-d6</td>
<td>2.0829mg/10ml</td>
<td>208.29</td>
<td>1 mM</td>
<td>1 µM</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

The working concentrations contain all three internal standards of TMAO, ADMA and SDMA that are 10 µM of TMAO-d9, 1 µM of ADMA-d7 and SDMA-d6. All are combined in one mixture with methanol as the solvent. Working stocks are prepared along with every batch meaning on each day.

This is an isotope dilution method, in which an isotope analog (deuterated in this case) of the analytes are used as internal standards. Internal standards in methanol are added to the calibration standards as well as to the unknown samples to be analyzed. Calibration curves are run with every batch of samples meaning sample specimen run per each day.

80 µl of this working concentration in methanol is added to the 20 µl of calibrators or quality control samples or to the specimen samples.

C. Quality control samples:
Pooled samples are used to confirm the batch level quality control of the samples. Normal healthy volunteer patient (plasma/urine), kidney disease patient (plasma/urine) (CKD), end stage renal disease (ESRD) patient (plasma) are used for the quality assurance purposes. They are prepared identically with and along with the unknown samples for accuracy and precision of that particular batch.

The calibration curve is done for each batch of samples along with the unknowns. In mass spectrometry, quality control steps are at various levels of the instrument, nitrogen tank change, regular rough oil change, column wash before and after the batch run to prevent carry over, purging the LC every time before the batch starts to avoid the noise in the run, cleaning curtain plate every day for the clean peaks. Observing the peak quality, signal to noise ratio are also are the things to consider for the quality assurance, accuracy, sensitivity and the precision of the instrument and thus the results.
Quality control samples are run for every batch. A batch is defined as the number of samples prepared and run per day. A typical run if about 50 samples per day per batch. Quality control samples are run after the 9 calibrators and then specimen samples are run after.

The criteria of acceptance of a batch is based on the calibrators and the quality control samples by reviewing their peak integrity and the CV +/- 15%.

6. Methods:

A. Liquid chromatography mass spectrometry Configuration
   i. Configuration
   **HPLC system:** Shimadzu prominence LC system  
   **Mass Spectrometry:** API 4000 triple quadruple mass spectrometer (Q Trap)  
   **Separating column:** Luna Silica column (150 mm x 2.1 mm, 3 µm particle size) (phenomenex)  
   **Guard Column:** 4 mm x 2.1 mm silica filter  
   **Source:** ESI source (TURBO SPRAY)

   ii. Auto sampler:
   a. Model : SIL-20 A/HT  
   b. Raising vol: 200 µl  
   c. Needle Stroke: 52 mm  
   d. Rinsing Speed: 35 µl/Sec  
   e. Sampling Speed: 15 µl/sec  
   f. Purge Time: 25 min  
   g. Rinse dip time: 0 Sec  
   h. Rinse mode: After Aspiration  
   i. Control Vial Needle stroke: 52 min  
   j. Total duration: 11.966 min

   iii. Pumps: Isocratic gradient
   a. Pumping Mode : Binary Flow  
   b. Total Flow : 0.2 ml/min  
   c. Pump B conc : 90 %  
   d. Pump A conc: 10 %  
   e. Mobile phase C for needle washing.  
   f. Pressure Limits (Pump A & B)  
   g. Minimum : 0 Psi  
   h. Maximum: 3500 Psi

   iv. Acquisition Method:
   a. Duration : 12.000 min  
   b. Synchronization mode: LC Sync  
   c. Instrument: API 4000  
   d. Ion source: Turbo Spray  
   e. Device Method: Shimadzu LC controller method
v. Mass Spec (MS):
   a. Scan Type: MRM
   b. Polarity: Positive

Period Summary:
Duration: 11.966min
Delay time: 0

Table 6: Parent (Q1) and daughter ions (Q3) of each analyte is shown here in the table-

<table>
<thead>
<tr>
<th></th>
<th>Q1 Mass (Da)</th>
<th>Q3 Mass (Da)</th>
<th>ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>76</td>
<td>58</td>
<td>TMAO</td>
</tr>
<tr>
<td>2</td>
<td>202.900</td>
<td>46</td>
<td>ADMA</td>
</tr>
<tr>
<td>3</td>
<td>203.1</td>
<td>172</td>
<td>SDMA</td>
</tr>
<tr>
<td>4</td>
<td>76</td>
<td>59</td>
<td>TMAO-2</td>
</tr>
<tr>
<td>5</td>
<td>85</td>
<td>66</td>
<td>TMAO-d9</td>
</tr>
<tr>
<td>6</td>
<td>210.1</td>
<td>77.2</td>
<td>ADMA-d7</td>
</tr>
<tr>
<td>7</td>
<td>209.2</td>
<td>70.00</td>
<td>SDMA-d6</td>
</tr>
</tbody>
</table>

The compounds are ionized in the electron spray ionization operated in the positive mode. Ionizing voltage is 5500 V, and ion source temperature 650 °C. Collision gas: 7, curtain gas: 20, GS1 60, GS2: 50. The total ion current chromatograms are obtained by a mass spectrometer in multiple reactions monitoring mode (MRM). The ion pairs used for the quantitative analysis are m/z 76 → 59 for TMAO, m/z 85 → 66 for TMAO-d9, m/z 202.9 → 70 for ADMA, m/z 210.1 → 77.2 for ADMA-d7, m/z 203.1 → 70 for SDMA and m/z 209.2 → 116.1 for SDMA-d6. Data is collected and analyzed using Analyst 1.6 software (AB Sciex, Canada).

Regression analysis is performed by using the instrument software (Analyst version 2.1 see the screenshots below) for all solutes (TMAO, ADMA and SDMA) for a linear plot, which is used for the measurement of molecules in the sample. Calibration curves are run every batch of samples at the start. Replicate data point CV should be less than 15%.

B. Methods used in the Analyst software-

The software is divided into discrete functional areas called modes (figure 1). These modes allow to perform activities related to a main task. Modes are accessed through the Navigation bar or the Mode list in the toolbar. Switching from one mode to another can be done without any loss of work.

Different modes in ‘Analyst’ software are used for the building up the method, acquiring the data and for the data analysis of TMAO, ADMA and SDMA. Analyst software is in-built software for this LC-MS/MS instrument. The method that is used for measuring the compounds (TMAO, ADMA and SDMA) is shown below in a stepwise manner.
   i. Hardware configuration
   ii. Building Acquisition method and saving the method
i. **Hardware configuration:** Hardware configuration is to set up the connection between LC and MS through ‘Analyst’ software. Also the hardware configurations gives information about the status of vacuum, curtain pressure, exhaust, source pressure and the status of the analyzer and interlace turbo pumps.

A specific hardware profile is to perform the analysis and to establish the link between LC and MS.

![Image of Analyst software](image)

**Figure 1.** Mass spec computer desktop→Analyst software icon double click→Modes present at left side

Methods used to acquire samples run and quantitate the data:

- Acquisition method.

ii. **Building Acquisition Method (Multiple Reaction Monitoring - MRM):**

The acquisition method used to measure the analytes, TMAO, ADMA and SDMA is shown in the figures 2-6. This method is used to acquire the samples in the analyst software.
Figure 2. Analyst icon ➔ Acquire mode ➔ build acquisition method ➔ mass spec

Figure 3. Analyst icon ➔ Acquire mode ➔ build acquisition method ➔ mass spec ➔ advanced MS
Figure 4. Analyst icon→Acquire mode→build acquisition method→Shimadzu LC system→equilibrate→pumps

Figure 5. Analyst icon→Acquire mode→build acquisition method→Shimadzu LC system→injection
Save the acquisition method by going to ‘file’, ‘save as’. The samples are acquired in the mass spectrometer by applying this acquisition method and the settings constant and not changed for this particular analytes’ measurement.

7. Sample Preparation from Plasma or Serum:

Steps of procedure performed before sample removing from the freezer and thawing:

a) Approximately 40 specimen samples are prepared at once for one day.

b) Empty tubes are arranged in the Eppendorf rack. The calibrators and QC tubes are labeled. The 40-50 specimen samples that are to be analyzed for the day are checked for agreement with the manifest table, the child sample IDs are prepared for them.

c) Child barcode labels are generated (discussed in detail in SOP_sample handling document) with the help of lab matrix software which operates in secure research environment (SRE) by keeping the original/parent sample IDs or Biocon IDs same and by adding suffix ‘.1’ to the parent sample ID. Example of parent and child sample IDs are ‘Bioconabc1’ and ‘Bioconab1.1’ respectively.

d) These child sample IDs are printed from the lab matrix with the help of zebra printer (that print the sample IDs on labels) and the labels are affixed.
to the empty eppendorf tubes into which the biomaterial (serum or urine) is aliquoted. Two copies of the child sample IDs are made, one copy for the aliquoting the sample and the other copy for the auto sampler vial.

Steps of procedure performed after sample removing from the freezer and thawing:

e) The tubes are arranged with an empty space between each tube for better air circulation at room temperature.

f) The tubes should be cold to touch after the samples are thawed. This process generally takes 15 min depending on the sample volume.

g) After thawing, vortex the tubes for 10 sec.

h) Centrifuge the tubes at 4 °C at 1000 g for 1 min in the centrifuge (Eppendorf 5417R Rotor FA 45-30-11).

i) Aliquot 20 µl of serum/plasma sample into snap lock, spill proof Eppendorf tubes (Dot Scientific Inc. - catalog no: 607-GMT).

j) To the 20 µl of plasma or serum sample in the child barcode labeled Eppendorf tube, 80 µl of internal standard mixture (containing 10 µM TMAO-d9, 1 µM of ADMA-d7 and 1 µM of SDMA-d6) in methanol is added as this is an isotope dilution method.

k) The working internal standard solution mixture is made in methanol solvent. Hence by adding the internal standard mixture to the plasma leads to the precipitation of all the proteins present in it leading to protein denaturation of all the proteins present in the serum/plasma/urine.

l) Then by vortexing using multi tube vortex (speed 1500 for 2 min) the sample thoroughly for 2 min followed by centrifugation at 20,000 g for 10 min at 4 °C, the denatured proteins in the pellet is carefully separated from the supernatants before the pellet discarded.

m) While the samples are vortexing, auto sampler vials are placed in empty auto sampler vial rack. The child sample ID labels (that are printed already 2 copies before specimens taking out of the freezer) are pasted on to them.

n) The supernatant is transferred into the auto sampler vial by cross checking visually the eppendorf tube to the auto sampler vial label and then subjected to LC-MS/MS analysis.

8. Samples Preparation from Urine

Steps before thawing the samples:

a) Approximately 40 specimen samples are prepared in the batch for one day.

b) Empty tubes are arranged in the Eppendorf rack. The calibrators and QC tubes are labeled. The 40-50 specimen samples that are to be analyzed for the day are checked for agreement with the manifest table, the child sample IDs are prepared for them.

c) Child barcodes labels are created by the lab matrix software by adding suffix ‘.1’ to the parent or original sample IDs printed through zebra printer (details provided in SOP_sample handling document) and pasted on to the empty tubes in which the biomaterial is placed.
Steps after thawing the samples:

d) The urine specimen tubes, calibrators and QC samples are arranged in tube racks with an empty space between each tube for better air circulation at room temperature. The tubes should be cold to touch after the samples are thawed. This process generally takes 15 min depending on the sample volume.

e) After thawing at room temperature, vortex the tubes for 10 sec.

f) Centrifuge the tubes at 4 °C at 1000 g for 1 min in the centrifuge (Eppendorf 5417R Rotor FA 45-30-11).

g) Prepare a 40 times dilution aliquot: Aliquot 20 µl of urine sample to 780 µl HPLC grade water containing snap lock, spill proof Eppendorf tubes with matching labels (Dot Scientific inc- catalog no: 607-GMT).

h) Vortex the tubes for 15 sec.

i) Centrifuge the tube for 1 min at 4 °C at 1000 g.

j) Aliquot the 20 µl of diluted urine sample into the fresh Eppendorf tubes with child barcode labels.

k) To this 80 µl of working internal standard solution in methanol (as described above for plasma/serum preparation) is added to the sample volume. Since an analog of analyte is used as an internal standard (that is deuterated TMAO, ADMA and SDMA), the method is an isotope dilution method to measure the specific analytes. This step also serves as a protein denaturation step.

l) Then the mixture is vortexed for 2 min, centrifuged at 20,000 g for 10 min at 4 °C.

m) While the samples are vortexing, auto sampler vials are placed in empty auto sampler vial rack. The child sample ID labels (that are printed already 2 copies before specimens taking out of the freezer) are pasted on to them.

n) As the denatured protein precipitated into the pellet, 60 µl of supernatant carefully drawn off and collected into auto sampler vial with insert by visually cross checking the auto sampler vial label against the sample tube label.

o) 5 µl of sample prepared in the auto sampler vial is subjected to LCMSMS analysis.

9. Determination of TMAO, ADMA and SDMA by LC-MS/MS.

Acquisition method settings are not changed. Upon preparing the samples, the samples are ready to scan to measure the analytes.

A. Order of samples checked into the Analyst software:
A batch of samples (specimens, calibrators and QC samples) are prepared for each day. The prepared samples in auto sampler vials labeled with child barcodes are transferred to mass spectrometer tray/rack.

Calibrators are placed in the first 9 slots of the rack, next QC samples (2) and then specimen samples (40-50) are placed in the rack.

First the mass spectrometer scans the calibrators’ next QC samples and then specimen samples in an order. More detailed description of analyst software operation to run the samples is given below.

5 µl of sample from the auto sampler vial in methanol is subjected to LC-MS/MS analysis. LC-MS/MS system used here is Shimadzu prominence LC system coupled to an API 4000 triple quadruple mass spectrometer (AB Sciex, Canada).

The main column used here is from Phenomenex Luna Silica column (150 mm x 2.1 mm, 3 µm particle size) that is guarded by guard column (4 mm x 2.1 mm silica filter) at room temperature.

The mobile phases (Table 1) used here are, mobile phase ‘A’ (consists of Water with 0.1 % Propionic acid), mobile phase ‘B’ (consists of Methanol with 0.1 % acetic acid) and mobile phase ‘C’ (consists of 50 % methanol with 0.2 % Formic acid) delivered with scheduled binary flow with a flow rate of 0.2 ml/min.

B. Analyst Software in detail: subjecting the samples for LCMSMS analysis.

i. Building acquisition batch and saving the batch

ii. Equilibration of mass spectrometer and the starting the run

i. Building an acquisition batch file:
Path: Analyst-navigation bar-Acquire-Build acquisition batch (figure 7- blue ring)

Building an acquisition batch is achieved by double clicking the ‘build acquisition batch’, a new window appears (figure 8), selecting ‘add set’ shown in blue ring of figure 8 then selecting ‘add samples’ as shown in blue ring in figure 9. A new window pops up by selecting the ‘add samples’ tab as shown in figure 10, in which typing the number of samples to be listed is added in the space given shown in blue ring. Further clicking OK gives the number of samples required and added with the names, for example of 5 samples, sample 001, sample 002, sample 003, sample 004 and sample 005 shown in figure 11. And in each row, the sample name is changed to the sample id by simply placing the cursor in the row, delete and then add new sample name for example STD1, STD2, STD3 etc., as shown in figure 12 and finally saving the built acquisition file by going to ‘file’ and clicking ‘save as’ to give the name with (date of acquired) ‘MM/DD/YY_Biocon’ (the study name) for the saved file. Each sample is named with its sample IDs under the file name saved as above.

A typical batch run per day consists of approximately 50 samples, 9 calibrators, 2 quality controls and remaining specimen samples are under one batch file name.

Vial position numbering is important. Placing the standards, samples and quality controls auto sampler vials in the auto sampler rack very carefully and cross checking visually the vial positions inside the ‘analyst’ software is key. Then the file is saved with the new file name. This order of entering the sample id barcodes into ‘Analyst’ is through
the bar code reader scanned directly by comparing them from auto sampler vial rack (discussed in sample handling document).
After saving the file, to run the batch file, select ‘submit’ button for the samples to get in the line (figure 13).

Figure 7. Analyst icon→Acquire mode→build acquisition batch
Figure 8. Analyst icon ➔ Acquire mode ➔ build acquisition batch ➔ add set
Figure 9. Analyst icon ➞ Acquire mode ➞ build acquisition batch ➞ add set ➞ add samples
Figure 10. Analyst icon ➔ Acquire mode ➔ build acquisition batch ➔ add set ➔ add samples ➔ type number ➔ OK
Figure 11. Analyst icon ➔ Acquire mode ➔ build acquisition batch ➔ add set ➔ add samples ➔ type number ➔ OK ➔ samples shown
Figure 12. Analyst icon → Acquire mode → build acquisition batch → add set → add samples → type number → OK → change the name with a specific name.

Figure 13. Analyst icon → Acquire mode → build acquisition batch → add set → add samples → type number → OK → change the name with a specific name → Go to 'submit' tab at the tab → click 'Submit'.
ii. Equilibration of mass spectrometer and the starting the run: Upon the submission of the samples, to view the queue of samples lined for acquisition push the ‘view queue’ icon (blue ring) as shown in figure 14. Further equilibrate the system by pushing ‘equilibrate’ icon (blue ring) on the navigation bar shown in figure 15 and further click the “start sample” button (blue ring) to actually start the sample as shown in figure 16.

Figure 14. Analyst icon → Acquire mode → build acquisition batch → add set → add samples → type number → OK → change the name with a specific name → Go to 'submit' tab at the tab → click 'Submit' → click 'view queue' icon to see the samples waiting in line.

Figure 15. Analyst icon → Acquire mode → build acquisition batch → add set → add samples → type number → OK → change the name with a specific name → Go to 'submit' tab at the tab → click 'Submit' → click 'view queue' icon to see the samples waiting in line → on the tool bar click 'equilibrate' icon as shown

Figure 16. Analyst icon → Acquire mode → build acquisition batch → add set → add samples → type number → OK → change the name with a specific name → Go to 'submit' tab at the tab → click 'Submit' → click 'view queue' icon to see the samples waiting in line → on the tool bar click 'equilibrate' icon as shown → click the 'start sample' icon to start running the samples in an order

a. To view the raw data file, the following path is pursued:
b. To go to the analyte ions, the path is: Analyst → file → open data file → choose the specific data file → OK → shows the raw data of data file opened → tool bar → explore → extract ions → choose the specific analyte in the small window popped.

c. To go to the specific analyte peak, here TMAO peak. The path is Analyst → file → open data file → choose the specific data file → OK → shows the raw data of data file opened → tool bar → explore → extract ions → choose the specific analyte, TMAO here.

j. **Quantification of data results:**

   Double click ‘build quantification method’, a new window pops up to choose the sample (here standard) and select ‘OK’ (figure 17). Figure 18, shows the assigning parent and daughters ions for the analytes and the internal standards. Figure 25 shows the integration done for a specific analyte, TMAO taken as an example. Set the concentration units as ‘µM’ (blue ring), smoothing width as ‘3’ (orange ring). Further set up background parameters by first selecting the back ground region other than peak and choose the icon as shown in figure 20. Then select the peak region first and then select icon ‘select peak’ as shown in figure 21. To save the changes say ‘apply’ as shown in orange ring.

   In the ‘calibration’ tab, do check mark in the box to apply changes to all analytes, ‘apply to all analytes’ (figure 22, blue square), set the fit as ‘linear’ in the drop down menu (figure 22, orange square) and weighting to ‘1/(x*x)’ (figure 23, blue square) and finally go to ‘file’ ‘save as’ to save the quantification file (Figure 24).

   These below quantification settings are constant for measuring all the analytes.
Figure 17. Path: Analyst→left side→ choose quantitate mode→build quantitation method→choose the data file that needs to be quantified→select ‘OK’

Figure 18. Path: Analyst→left side→ choose quantitate mode→build quantitation method→choose the data file that needs to be quantified→choose the components→parent and daughter ions of analytes and internal standards.

Figure 19. Path: Analyst→left side→choose quantitate mode→build quantitation method→choose the data file that needs to be quantified→choose the components→parent and daughter ions of analytes and internal standards→integration→set smoothing width of '3' points→choose the 'analyte'
Figure 20. Path: Analyst→left side-choose quantitate mode→build quantitation method→choose the data file that needs to be quantified→choose the components→parent and daughter ions of analytes and internal standards→integration→set smoothing width of '3' points→choose the 'analyte'→set the parameters for back ground noise.
Figure 21. Path: Analyst → left side-choose quantitate mode-build quantitation method → choose the data file that needs to be quantified → choose the components-parent and daughter ions of analytes and internal standards → integration → set smoothing width of '3' points → choose the 'analyte' → set the parameters for background noise → choose the actual analyte peak → click 'apply'.

Figure 22. Path: Analyst → left side-choose quantitate mode-build quantitation method → choose the data file that needs to be quantified → choose the components → parent and daughter ions of analytes and internal standards → integration → set smoothing width of ‘3’ points → choose the 'analyte' → set the parameters for background noise → choose the actual analyte peak → click 'apply' → set 'linear' fit → check the box of the apply to all analytes.
Figure 23. Path: Analyst → left side → choose quantitate mode → build quantitation method → choose the data file that needs to be quantified → choose the components → parent and daughter ions of analytes and internal standards → integration → set smoothing width of ‘3’ points → choose the 'analyte' → set the parameters for background noise → choose the actual analyte peak → click 'apply' → set 'linear' fit → check the box of the apply to all analytes → weighting as 1/(x*x).

Figure 24. Path: Analyst → left side → choose quantitate mode → build quantitation method → choose the data file that needs to be quantified → choose the components → parent and daughter ions of analytes and internal standards → integration → set smoothing width of ‘3’ points → choose the 'analyte' → set the parameters for background noise → choose the actual analyte peak → click 'apply' → set 'linear' fit → check the box to the apply to all analytes → weighting as 1/(x*x).
k. Saving Data results:

The quantification method created (discussed in method section) is applied for the data acquired for all the samples (calibrators, QC and the Specimens).

Saving the data results file with data of acquired the data with the study name to ‘MMDDYY_Study name’ to differentiate with other files.

By following the above path, by providing specific quantification method for the analytes, data results appear in a new window as shown in figure 25. Save the results file by going to ‘file’ and further clicking ‘save’. The calibration curve of the specific chosen analyte, is created by clicking the icon as showed in figure 26 (blue ring).

Figure 25. Path: Analyse→navigation bar→quantitate→quantitation wizard→choose files→choose quantification method→finish→data results→file-save as→save the file.

Figure 26. Path: Analyte-navigation bar-quantitate→quantitation wizard→choose files→choose quantification method→finish→data results→tool bar→choose the graph to see the calibration curve.

Steps to accept the data after quantitation:

1) Evaluate run performance looking at the accuracy for the calibrators and QC samples +/- 15%.

2) Review peak integration and Signal to noise ratio of each peak.

3) The acceptance criteria would be QC should be within 15% of the accepted/validated values or within 2SD of the accepted mean.
10. Data management, transfer and storage:

A. Data management and transfer: Data transfer and data management is through lab matrix, which is explained in detail in SOP_sample handling document.

Briefly, data results from the Analyst software is exported in the 'CSV' file that is further results file is uploaded into the lab matrix software and then the data results with all parameters (such as subject id, biomaterial type, freezer location etc.) can be downloaded from the lab matrix. The analyte results with combined information from Analyst and Lab matrix will be further sent to SDCC (This process is discussed in detail with screen shots in SOP sample handling file).

B. Data storage: 3 storage places.
   1) Analyst software of mass spectrometry computer.
   2) Also the mass spectrometry computer is constantly backed up with Clash Plan software
   3) Data results are also stored in our inventory software, Lab matrix.

The data is initially delivered in the Analyst software that is connected directly to LC/MS/MS instrument. The resulting data in the Analyst software is exported into the excel spreadsheet and the excel spread sheet is transferred to the lab matrix software.
Lab matrix software has the query tool (Qiagram) that links the data results to the other parameters like freezer location, subject id, left over volume, biomaterial type etc. Hence the results are stored and also can be transferred to the data co-ordination center (SDCC). The data is also stored in Lab matrix after the retrieving from the Analyst software; the lab matrix operates in Secured Research Environment (SRE). This ‘lab matrix’ tool is important as it combines the freezer location, the remaining volume after aliquoting and the measured analyte concentrations’ results all linked together in one place.

Also the data from the mass spec computer (API 4000-PC) is backed up constantly by Crash Plan 12 Cloud software (picture below). Crash Plan runs continuously on the computer and backs up constantly while it is connected to the Internet. When the computer is off, back up will resume where it left off when it is turned back on. If the computer has not connected to the backup server for >3 days, an email notification is received notifying that there may be a problem with the backup. This CrashPlan is supported by Case Western Reserve University Utech services as shown in the picture below.