# Method Validation Report for a Selected Subset of 4 Phosphatidylethanolamine (PtdEtn) and 4 Plasmenylethanolamine (PlsEtn) Species using Stable Isotope Dilution, Flow Injection Tandem Mass Spectrometry

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## 1.0 *Method Summary*

The following report describes the materials and procedures to be used to quantitate 4 phosphatidylethanolamines (PtdEtn) and 4 plasmenylethanolamine (PlsEtn) glycerophospholipids species with the following sn-1/sn-2 compositions in serum: PtdEtn 16:0/18:3, 18:0/20:5, 18:0/22:4, 16:0/22:6 and PlsEtn 16:0/18:2, 18:0/20:5, 16:0/22:4, 16:0/22:6 Analytes are to be extracted from serum into ethylacetate using a pH adjusted liquid/liquid extraction procedure. Analyte intensities are measured by direct injection of the extraction solution into an Ionics 3Q tandem mass spectrometer operating in the negative ionization atmospheric pressure chemical ionization (APCI) mode. For PtdEtn analytes, the (M-H)- ion is selected by Q1 to be fragmented in the collision cell and the sn-1 (R-COO-) ion is selected by Q3 and used as the daughter ion. The exception is PtdEtn 18:0/20:5 where the sn-2 (R-COO-) ion is used for detection. For PlsEtn analytes the (M-H)- ion is selected by Q1 to be fragmented in the collision cell and the sn-2 (R-COO-) ion is selected by Q3 and used as the daughter ion for detection. Analytes are quantitated using a standard stable isotope dilution protocol where a constant amount of a non-endogenous stable isotope ($^{13}C_{19}$-PtdEtn 16:0/22:6 and $^{13}C_{6}$-PlsEtn 16:0/22:6) is added to all unknown serum samples. The peak height ratio of each of the 4 PlsEtn and 4 PtdEtn species respective to the stable isotope is then determined. Serum concentrations are established by generating 4 standard curves where the y-axis is defined by isotope ratio and the x-axis is defined by concentration. Analytical standards of endogenous $^{12}C$ as well as standards of PtdEtn 16:0/18:3, PtdEtn 16:0/22:6, PlsEtn 16:0/18:2, and PlsEtn 16:0/22:6 are spiked into fetal bovine serum at various concentrations spanning >95% of the expected human concentration range. Serum concentrations are calculated by converting the observed isotope ratio into concentration using the isotope ratio:concentration relationship described by the standard curve.
Method performance characteristics of recovery (95-115%), accuracy (97-113%), and precision (9-12% within run and 10-13% between run) are such that the method meets basic validation criteria for investigational, case-control, regulatory-exempt, research use.

## 2.0 *Materials required*

- *Pipettes* – Eppendorf Reference Pipettes, 20µL, 100µL, 200µL, 1000µL (Eppendorf; Hamburg, Germany)
- *Centrifuge* – Sufficient size to centrifuge two 96 well plates at 2000RCF
- *Bottle top Dispenser* – ChemSaver™ bottle top dispenser or equivalent (1-5 mL or 0.5-2.5 mL) (Brinkmann Instruments Inc; Westbury, NY, USA)
- *20µL Barrier Pipet Tips* – VWR - Cat#: 87001-694 or equivalent
- *Matrix 1.4mL Polypropylene Round Bottom Tubes*- (Fisher Scientific) Cat#50823880 or equivalent
- *Matrix 1.4mL Rack* – (Thermo Fisher); Cat# 50823921 or equivalent
- *Matrix Cap Strips (EVA)* – (Thermo Fisher); Cat# 50823825 or equivalent

- *Vortex Mixer* – Vortex-Genie I™ (Scientific Industries Inc; Bohemia, NY, USA) or equivalent
- *Eppendorf Mixmate Vortex Mixer* – VWR Cat# 14900-548 or equivalent
- *Thermo Scientific Unmarked Analysis Tubes* (Fisher Scientific) Cat# AB4170 or equivalent
- *Thermo Scientific Matrix SepraSeal Capping System; Pre – Split; Piercable; Autoclavable Caps* (Fisher Scientific) Thermo No. 4465BLU
- *HPCL Glass Vial* – Chromatographic Specialties, Cat# CS51820714
- *HPCL Vial Cap* – Chromatographic Specialties, Cat# CS51820717

## Solvents:

- *Ethyl Acetate* – Optima Grade, cat. E196-4, (Fisher Scientific) or equivalent
- *Formic Acid* – 98-100% R.G, Reagent Grade ACS, cat. 33015-500mL, (Sigma Aldrich Canada Ltd.) or equivalent
- *Ultrapure Water* – HPLC Grade Water, cat. W7-4 (Fisher Scientific) or equivalent
- *Dichloromethane* – VWR Cat# BDH1113-4LG
- *Fetal Bovine Serum* – Gibco

## Standards:

- PPI-1050 – 2mg/mL C13 stable isotope labeled plasmalogen ethanolamine 16:0/22:6 prepared in DCM and procured from PDI
- PPI-1052 – 3mg/mL C13 stable isotope labeled phosphatidyl ethanolamine 16:0/22:6 prepared in DCM and procured from PDI
- PPI-1040 – 3mg/mL plasmalogen ethanolamine 16:0/22:6 prepared in DCM and procured from PDI
- PPI-1049 – 3mg/mL phosphatidyl ethanolamine 16:0/22:6 prepared in DCM and procured from PDI
- PPI-1058 – 2mg/mL plasmalogen ethanolamine 16:0/18:2 prepared in DCM and procured from PDI
- PPI-1059 – 2mg/mL phosphatidyl ethanolamine 16:0/18:3 prepared in DCM and procured from PDI

## Standards structures:

**PPI 1040**
$C_{43}H_{72}NO_6P$; Exact Mass: 729.50973

**PPI 1049**
$C_{43}H_{72}NO_7P$; Exact Mass: 745.5046

**PPI 1050**
$C_{37}{}^{13}C_{6}H_{72}NO_{6}P$; Exact Mass: 735.52985

**PPI 1052**
$C_{24}{}^{13}C_{19}H_{72}NO_{7}P$; Exact Mass: 764.56838

**PPI 1059**
$C_{39}H_{70}NO_{7}P$; Exact Mass: 695.4890

**PPI 1058**
$C_{39}H_{72}NO_{6}P$; Exact Mass: 681.5097

| PPI # | Structure _ Closed Ring | MS/MS Transition _ Open Ring |
|---|---|---|
| 1059 | | 712.5 / 255.2 |
| 1049 | | 762.5 / 255.2 |
| 1058 | | 698.5 / 279.2 |
| 1040 | | 746.5 / 327.2 |
| 1050 | | 752.5 / 327.2 |
| 1052 | | 781.5 / 271.2 |

## 3.0 *LC-MS/MS conditions*

*Mass Spectrometer* - Ionics 3Q triple quadrupole mass spectrometer equipped with a negative APCI source.
*Customized GX-271*- Customized Gilson GX-271liquid handler equipped with automated sample injection and solvent delivery pump.

- LC flow rate: 0.4ml/min

- Mobile phase: 4% water in ethyl acetate (EtOAc)
- Acquisition time: 0.5min/sample
- MS Source Parameter:

| Description | Value |
|---|---|
| Scan Type | MRM |
| Polarity | Negative |
| Drying Gas Temperature | 100 ºC |
| Nebulizer Gas Temperature | 490 ºC |
| Heating Gas Temperature | 350 ºC |
| Curtain Cap Voltage | -1000 V |
| HSID Temperature | 200 ºC |
| Source Temperature | 495 ºC |
| Entrance Voltage | -50 V |
| Detector Deflector | 300 |
| Detector Voltage | 2600 V |
| Collision pressure | 430 |
| Corona Discharge | -4 |
| Collision Cell Lens 2 | 100 |
| Dwell time | 50 msec |

## 3.1 10MRMs analysis transition:

| | Metabolite name | Molecular formula | Parent mass | Daughter mass |
|---|---|---|---|---|
| 1 | PlsEtn 16:0/22:6 | $C_{43}H_{74}NO_7P$ | 746.5 | 327.2 |
| 2 | 13C_PlsEtn 16:0/22:6 | $C_{37}{}^{13}C_6H_{74}NO_7P$ | 752.5 | 327.2 |
| 3 | PtdEtn 16:0/22:6 | $C_{43}H_{74}NO_8P$ | 762.5 | 255.2 |
| 4 | C13_ptdEtn 16:0/22:6 | $C_{24}{}^{13}C_{19}H_{74}NO_8P$ | 781.5 | 271.2 |
| 5 | PtdEtn 16:0/18:3 | $C_{39}H_{72}NO_8P$ | 712.5 | 255.2 |
| 6 | PlsEtn 16:0/18:2 | $C_{39}H_{74}NO_7P$ | 698.5 | 279.2 |
| 7 | PtdEtn 18:0/22:4 | $C_{45}H_{82}NO_8P$ | 794.5 | 283.2 |
| 8 | PtdEtn 18:0/20:5 | $C_{43}H_{76}NO_8P$ | 764.5 | 301.2 |
| 9 | PlsEtn 16:0/22:4 | $C_{43}H_{78}NO_7P$ | 750.5 | 331.2 |
| 10 | PlsEtn 18:0/20:5 | $C_{43}H_{76}NO_7P$ | 748.5 | 301.2 |

# 4.0 *Sample preparation*

## 4.1 Preparation of stock ring-closed $C^{13}$ stable isotope internal standard solution

PPI-1050: $C_1V_1=C_2V_2$

$(2mg/mL)\ V_1 = (60\mu g/mL)(1000\mu L)$

$V_1 = 30\mu L$

PPI-1052: $C_1V_1=C_2V_2$

$(3mg/mL)\ V_1 = (150\mu g/mL)(1000\mu L)$

$V_1 = 50\mu L$

1. Aliquot 1000µL DCM into a clean 2mL microcentrifuge tube.

2. Remove 50µL of DCM followed by an additional 30µL of DCM from the tube.
3. Into the remaining 920µL of DCM, aliquot 50µL of PPI-1052 and 30µL of PPI-1050. Due to the volatility of DCM, aliquoting the standard into the DCM helps minimize evaporation of the solution.
4. Cap and vortex thoroughly.

## 4.2 Preparation of ring-closed $C^{13}$ and $C^{12}$ standards stock solution (CC8)

PPI-1040: $C_1V_1=C_2V_2$

$(3mg/mL)\ V_1 = (400\mu g/mL)(2000\mu L)$

$V_1 = 266.6\mu L$

PPI-1049: $C_1V_1=C_2V_2$

$(3mg/mL)\ V_1 = (600\mu g/mL)(2000\mu L)$

$V_1 = 400\mu L$

PPI-1058: $C_1V_1=C_2V_2$

$(2mg/mL)\ V_1 = (200\mu g/mL)(2000\mu L)$

$V_1 = 200\mu L$

PPI-1059: $C_1V_1=C_2V_2$

$(2mg/mL)\ V_1 = (100\mu g/mL)(2000\mu L)$

$V_1 = 100\mu L$

1. Aliquot 2000µL of DCM into a clean 15mL falcon tube.
2. Remove volumes of DCM equivalent to the volumes of standards to be aliquoted from the falcon tube into a glass vial.
3. Aliquot 266.6µL of PPI-1040, 400µL of PPI-1049, 200µL of PPI-1058, and 100µL of PPI-1059 into the glass vial containing 1033.4µL of DCM (total volume: 2mL). This stock solution will be serially diluted to create the external calibration standards, see table below. Store each standard in an HPLC vial.

The following table represents the concentration of each standard at each calibration curve point:

| Calibrator Stock solution | Volume | DCM (uL) | Final Volume (uL) | PPI-1040 (ug/mL) | PPI-1049 (ug/mL) | PPI-1058 (ug/mL) | PPI-1059 (ug/mL) |
|---|---|---|---|---|---|---|---|
| CC8 | 2000uL | 0 | 1000 | 400 | 600 | 200 | 100 |
| CC7 | 1000uL of CC7 | 1000 | 1000 | 200 | 300 | 100 | 50 |
| CC6 | 1000uL of CC6 | 1000 | 1000 | 100 | 150 | 50 | 25 |
| CC5 | 1000uL of CC5 | 1000 | 1000 | 50 | 75 | 25 | 12.5 |
| CC4 | 1000uL of CC4 | 1000 | 1000 | 25 | 37.5 | 12.5 | 6.25 |
| CC3 | 1000uL of CC3 | 1000 | 1000 | 12.5 | 18.75 | 6.25 | 3.125 |
| CC2 | 1000uL of CC2 | 1000 | 2000 | 6.25 | 9.375 | 3.125 | 1.5625 |
| CC1 | N/A | N/A | N/A | 0 | 0 | 0 | 0 |

## 4.3 Preparation of the external calibration stock standards

These standard stock solutions will be used in each calibration standard extraction and will be stored at -20°C.

1. Label 8 ethyl acetate washed 1.4mL matrix tubes with the designations CC_01 – CC_08.
2. Aliquot 100µL of 4% formic acid prepared in HPLC grade water to each tube.
3. Aliquot 10µL of the prepared C12 standard stock solution into each tube using a new pipette tip for each aliquot.
4. Aliquot 10µL of the prepared C13 standard stock solution into each tube using a new pipette tip for each aliquot.
5. Aliquot 500µL of ethyl acetate to each tube.
6. Cap using EVA style caps, vortex at 1500RPM using the Eppendorf mixmate for 1 hour.
7. Centrifuge at 2500RPM for 2 minutes.
8. Aliquot 500µL of the organic fraction (top layer) from each tube into its own pre-labeled tube containing 19.5mL of ethyl acetate (40X dilution). Other working volumes are acceptable if the dilution factor is correct. This will be the stock solution for the calibration curve generation.

## 4.4 Preparation of ring-opened stable isotope internal standard solution (extraction solution bulk quantity)

The quantity prepared in the following steps will be enough for 20mL of extraction solution.

1. In a pre-washed (with ethyl acetate) 1.4mL matrix extraction tube, aliquot 100µL of previously prepared 4% formic acid (prepared in HPLC grade water).
2. Aliquot 10µL of the ring-closed standard solution into the tube containing the 4% formic acid.
3. Aliquot 500µL ethyl acetate into the 1.4mL tube containing the ring-closed standard and 4% formic acid.
4. Cap the tube using an EVA style cap.
5. Vortex using the Eppendorf mixmate for 1 hour at 1500RPM.
6. Centrifuge the 1.4mL tube for 2 minutes at 2500RPM.
7. Aliquot 500µL of the organic fraction (top layer) into a 50mL falcon tube. Dilute with 19.5mL of ethyl acetate. Total dilution factor of this solution (Solution A) is 40X.
    a. Solution A has the following concentrations of constituents:
        i. Formic acid: 0.1%.
        ii. Ring-opened standard PPI-1050: 0.03µg/mL, PPI-1050: 0.075µg/mL.

You can now begin the extraction of the curve and human serum samples.

## 4.5 Standard curve extraction preparation

A total of 200µL of FBS (Fetal Bovine Serum) is required for 8 calibrator curve points.

1. Pre-label eight 1.4mL pre-washed (with ethyl acetate and allowed to dry) matrix tubes.
2. Aliquot 20µL of FBS to each matrix tube.
3. Aliquot 100µL of water into each matrix tube (switching pipette tips after each aliquot).
4. Aliquot 750µL of each external calibration standard stock extraction solution into its respective labeled tube.
5. Cap each tube with the appropriate EVA style cap.
6. Vortex on the Eppendorf mixmate at 1500RPM for 1 hour.
7. Centrifuge at 2500RPM for 2 minutes.
8. Aliquot 200µL of the organic layer (top layer) into labeled analysis tubes and cap.

Once the solution preparations are complete, you can begin the following sample extraction procedure.

## 4.6 Sample extraction preparation

When transferring sera from the cryovial to the microcentrifuge tube, it is the technician's responsibility to cross check the accessioning number on the cryovial against the accession number on the side of the microcentrifuge extraction tube.

1. Aliquot 20µl of human patient serum into an ethyl acetate washed (and dried) and labeled 1.4ml matrix tube.
2. Aliquot 100µl of HPLC grade water into each extraction tube.
3. Aliquot 750µL of the internal calibration solution into each tube and cap with an appropriate EVA style cap. This should be performed on a row by row basis to prevent evaporation.
4. Place the rack into the Eppendorf mixmate and ensure it is secured. Vortex for 1hr at 1500RPM.
5. Centrifuge for 2mins at 2500RPM.
6. Transfer 200µl of organic layer (top layer) to a labeled corresponding 0.75mL analysis tube and cap with appropriate style caps.

Samples are now ready for analysis on the instrument.

## 4.7 Analysis run set up

To help prevent carry over, a series of blank samples are prepared. Each blank sample is 4% water saturated ethyl acetate and is approximately 700µL in volume to allow for triplicate injection.

1. Prepare enough blank samples to ensure that each patient sample prepared for analysis will be followed by an injected blank sample. Each blank sample will have enough volume to be injected 3 times. For example, if 60 patient samples are to be analyzed, a minimum amount of 20 blank samples are needed (plus the one initial blank). These blank samples will be positioned in an alternate rack than that of patient samples.

## 5.0 *Data analysis*

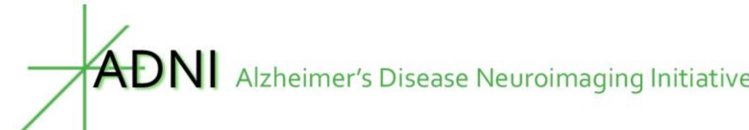
The Plasmalogen method is not a chromatography method. Classical peak integration strategies are not appropriate in that theoretical peak shape of the injected sample is square, not Gaussian. Accordingly, the appropriate measure of the amount of analyte present in a particular injection is best approximated by the average height of the square peak. Furthermore, since chromatography is not used, the concept of linear time is also not relevant in that there is no inherent relationship between scan 1 and scan 2 as would be present during peak elution where the rise in intensity is representative of the peak eluting and the decrease in intensity is representative of the elution finishing. Therefore, Phenomenome Discoveries Inc. (PDI) has developed Molana quantitation software to perform this kind of flow injection analysis by selecting the "S" curve function. Molana is a computer program that is able to convert data generated from the instrument into a universal file format, such as .mcxml format, and then calculate the 92-98$^{th}$ percentile peak height automatically. This eliminates the need for traditional peak picking optimizations such as data smoothing and peak and valley settings, manual peak review, and integration procedures. It is important that this automated process be initially validated against the manual process to ensure accuracy.

## 6.0 *Quality assurance*

### 6.1 Quality control samples

Three QC samples, containing Low, Medium and High DHA PlsEtn, respectively, are run along with patient samples in each batch to track the run acceptability. Based on the statistical analysis of 20 replicates of Low, Medium and High QC samples run in the front, and tolerance limits for each QC sample that should be within the range of True value ± Total error (Total error = Bias±2SD), the following criteria have been set for each QC sample:

- The DHA PlsEtn concentrations in the Low QC sample should be detected in the range of 3.9±1.7µM.
- The DHA PlsEtn concentrations in the Medium QC sample should be detected in the range of 8.1±2.7µM.
- The DHA PlsEtn concentrations in the High QC sample should be detected in the range of 12.7±4.2µM.



## 6.2 Curve acceptance

The following criterion constitutes an acceptable calibration curve:

- % deviance of actual vs. nominal values for all other standards of less than 20%.

If the calibration curve is not immediately acceptable, removal of no more than two standards from the standard curve and re-processing of the curve is allowed, as long as these points are not the LLoQ or the ULoQ. The removal of any curve point must be substantiated by means of an acceptable mathematical approach for removing outliers.

## 7.0 *Method validation*

## 7.1 Precision

Precision is determined in five replicates (within run) over two different days (between run) after spiking commercial serum samples with 3 different concentrations (final conc: 0.1, 0.5 and 2.0 µg/mL) of $^{13}C$-standard solutions of PlsEtn and PtdEtn. The within run CVs are 9-12% and the between run CVs are 10-13%. Within-run, between-run, between-day, and overall precision will be:

- Less than 20% RSD for low level sample.
- Less than 15% RSD for medium and high level samples.

Within run precision

| True value of $^{13}C$-standard conc (µg/ml) | % CV | |
|---|---|---|
| | $^{13}C$-PlsEtn | $^{13}C$-PtdEtn |
| 0.1 | 10% | 12% |
| 0.5 | 11% | 10% |
| 2 | 9% | 10% |

Between run precision

| True value of $^{13}C$-standard conc (µg/ml) | % CV | |
|---|---|---|
| | $^{13}C$-PlsEtn | $^{13}C$-PtdEtn |
| 0.1 | 12% | 13% |
| 0.5 | 11% | 10% |
| 2 | 10% | 11% |

## 7.2 Accuracy

Accuracy measures how close the test result is to the nominal value (concentration) of the analyte; it is described as a percentage of the test results compared with the nominal value.

Accuracy is determined by analysis of a minimum of five replicates per level at three concentration levels. The calculated mean at each concentration is compared to nominal values to determine the percent deviation of the mean from these nominal values, which serves as a measurement of the accuracy of the analytical method. Accuracy is:

- 97.2-109.1% for PtdEtn 16:0/18:3
- 105.3-113.1% for PlsEtn 16:0/18:2
- 100.8-106.1% for PtdEtn 16:0/22:6
- 99.9-101.4% PlsEtn 16:0/22:6.

The mean at each concentration level should be within 100±15% of their nominal concentrations, except for the Low concentration where the criteria is within 100±20%.

| Compound | Expected conc (μg/mL) | Average (μg/mL) | CV (%) | Accuracy (%) |
|---|---|---|---|---|
| PtdEtn 16:0/18:3 | 0.016 | 0.017 | 8.0 | 109.1 |
| | 0.063 | 0.067 | 9.2 | 105.8 |
| | 0.13 | 0.13 | 7.5 | 97.2 |
| PtdEtn 16:0/22:6 | 0.016 | 0.017 | 9.6 | 106.1 |
| | 0.063 | 0.065 | 8.1 | 103.4 |
| | 0.13 | 0.13 | 7.3 | 100.8 |
| PlsEtn 16:0/18:2 | 0.016 | 0.018 | 7.6 | 113.1 |
| | 0.063 | 0.069 | 6.2 | 110.3 |
| | 0.13 | 0.14 | 6.8 | 105.3 |
| PlsEtn 16:0/22 | 0.016 | 0.016 | 9.8 | 101.3 |
| | 0.063 | 0.063 | 7.4 | 99.9 |
| | 0.13 | 0.13 | 7.7 | 101.4 |

## 7.3 Recovery

Recovery measures the extraction efficiency of a given method within the defined limits of variability. It is determined by comparing the back-calculated extracted concentration to the nominal concentration, and expressed as a percentage. Although there is no absolute acceptance criterion for recovery, typically 70-130% recovery is required for LC-MS/MS method. Recovery is determined in five replicates after spiking serum control pools with 3 different concentrations of PtdEtn 16:0/18:3, PlsEtn 16:0/18:2, PtdEtn 16:0/22:6, and PlsEtn standard. The overall extraction recovery is:

- 95.0-117.5% for PtdEtn 16:0/18:3
- 105.3-115.2% for PlsEtn 16:0/18:2
- 100.1-109.5% for PtdEtn 16:0/22:6
- 95.8-114.9% PlsEtn 16:0/22:6.

| Compound | Expected conc (µg/mL) | Average (µg/mL) | CV (%) | Recovery (%) |
|---|---|---|---|---|
| PtdEtn 16:0/18:3 | 0.016 | 0.015 | 9.8 | 95.0 |
| | 0.063 | 0.070 | 5.0 | 111.3 |
| | 0.13 | 0.15 | 4.0 | 117.5 |
| PtdEtn 16:0/22:6 | 0.016 | 0.017 | 5.5 | 109.5 |
| | 0.063 | 0.064 | 4.0 | 102.8 |
| | 0.13 | 0.13 | 4.7 | 100.1 |
| PlsEtn 16:0/18:2 | 0.016 | 0.018 | 10.6 | 115.2 |
| | 0.063 | 0.067 | 7.1 | 109.3 |
| | 0.13 | 0.14 | 3.8 | 105.3 |
| PlsEtn 16:0/22:6 | 0.016 | 0.015 | 12.6 | 95.8 |
| | 0.063 | 0.072 | 7.7 | 114.9 |
| | 0.13 | 0.14 | 4.2 | 113.3 |