Phosphatidylethinal (PEth) Assay methods:

A 200 µL aliquot of whole blood was combined with 20 µL of a solution of internal standards, consisting of 0.5 µg/mL d_5-1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanol (d_5-PEth 16:0/18:2) and 0.5 µg/mL d_5-1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanol (d_5-PEth 16:0/18:1) as internal standards. This was next combined with 600 µL isopropanol (IPA) and 900 µL heptane, mixed, and centrifuged. The supernatant was dried under air and reconstituted in 50 µL heptane, 50 µL acetonitrile (ACN), and 50 µL IPA. The assay was determined to also be accurate when twice the volume of blood was precipitated with twice the volume of IPA and heptane but reconstituted to the same final volume. Extracts were filtered to remove particulate matter using 0.22 µm polypropylene filters, and 10 µL were injected onto an Agilent 1200 series liquid chromatograph coupled to an Agilent 6410 QQQ. Mobile phase A was 20% 2 mM ammonium acetate, pH 8.5, and 80% ACN. Mobile phase B was IPA. The analytical column was a Supelco Ascentis Express RP-Amide column, 150 mm x 2.1 mm, 2.7 µm particle size equipped with a C8 guard column. The elution gradient started at 10% B for 2 min, increased to 50% by 7 min, increased to 100% B by 9 min, held at 100% B until 15 min, returned to 10% B by 15.5 min, and equilibrated at 10% B for another 5 minutes before the next injection. The QQQ was operated in negative electrospray ionization mode with a capillary voltage of 5000 V; multiple-reaction monitoring consisted of 699.5 -> 279.3 *m/z* for 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanol (PEth 16:0/18:2), 701.7 → 281.3 *m/z* for 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanol (PEth 16:0/18:1), 704.5→ 279.2 *m/z* for d_5-PEth 16:0/18:2, and 706.7 → 281.2 *m/z* for d_5-PEth 16:0/18:1. The fragmentor voltages were 160 V for PEth 16:0/18:2 and d_5-PEth 16:0/18:2 and 145 V for PEth 16:0/18:1 and d_5-PEth 16:0/18:1, and the collision energies were 30 V for PEth 16:0/18:2 and d_5-PEth 16:0/18:2 and 36 V for PEth 16:0/18:1 and d_5-PEth 16:0/18:1. Extensive flushing of the injection needle was required to avoid carryover artifacts; the needle was washed for 5 sec after injecting and was washed again by allowing the mobile phase to flow through the needle for 30 sec after all analytes of interest had eluted. An 11-point standard curve was prepared by adding known quantities of unlabeled PEth 16:0/18:2 and PEth 16:0/18:1 to whole blood pre-tested to have low levels of phosphatidylethanols -- around those observed in a sample from a volunteer who abstained from alcohol consumption for 3 weeks -- and prepped identically to unknown samples. The standard curve ranged from the lower limit of quantification, which was 6.25 ng/mL for both PEth 16:0/18:2 and PEth 16:0/18:1, to 2.5 µg/mL. The peak height ratios of analyte/internal standard were fit to a second-order polynomial. Calculated concentrations of quality-control samples prepared each run had inter-run coefficient of variation of approximately 10% or less.