Phospholipid Analysis using LC-MS/MS

Extraction:

Phospholipids in mouse embryo eye lens tissues were identified using liquid chromatography-triple quadrupole tandem mass spectrometry (LC-MS/MS), employing an assay developed at the Integrated Lipidomics and Metabolomics Core Laboratory at Emory School of Medicine. Prior to LC-MS/MS analysis, phospholipids were extracted according to the Bligh and Dyer method (Bligh and Dyer, 1959). The eye lens tissues were homogenized with 500 µL of PBS in homogenizing tubes to obtain a homogeneous solution, followed by vortexing for 5 minutes at 4°C. The samples were then centrifuged at 13,000 rpm for 5 min at 4°C using an Eppendorf centrifuge 5810 R. All supernatants were transferred to 1.5 mL microcentrifuge tubes. To each tube, 1.2 mL of methanol and 600 µL of chloroform were added, and the tubes were vortexed for 30 minutes at speed 4 using a Fisher Scientific Multi-tube Vortexer. Subsequently, the samples were centrifuged at 4000 rpm for 10 minutes at 4°C, and the resulting supernatants were collected and transferred to clean 4 mL clear vials. The clear vials were weighed prior to the transfer of supernatants. To separate the organic and non-organic phases in the sample, 1 mL of 0.1M NaCl and 1 mL of chloroform were added. The samples were vortexed for 10 minutes at speed 4 and then centrifuged at 4000 rpm for 10 minutes. The samples were separated into a top layer (non-organic phase) which was discarded, and the lower layer (organic phase) was aliquoted separately using a Pasteur pipette and dried under nitrogen. The dried lipid weight was recorded, and the total weight was subtracted from the weight of the empty vial. The samples were stored at -80°C until ready for LC-MS/MS analysis. Prior to LC-MS/MS analysis, samples were reconstituted with 1 mL of 1:1 chloroform/methanol. Subsequently, 100 µL of sample was transferred into HPLC vials and capped with septa and analyzed by LC-MS/MS. The LC-MS/MS and chromatography parameters used for the phospholipids analysis are as follows:

Chromatography:

Phospholipid analysis was performed using an AB SCIEX QTRAP 5500 – Linear Ion Trap Quadrupole LC/MS/MS coupled to a Shimadzu EXION HPLC. Phospholipids from the extracted eye lens tissue were resolved on reverse-phase high performance liquid chromatography with a C18 column [Thermo Scientific Part No. 17126 – 104630, Dimensions (mm) 100 x 4.6, Accucore ™], using an 18-minute binary gradient. For chromatography, Solvent A consisted of 40:60 Water: Acetonitrile (ACN) with 10mM Ammonium Formate, and Solvent B consisted of 90:10 Isopropyl Alcohol (IPA)/ Acetonitrile with 10mM Ammonium Formate; both Solvent A and B contained 0.1% Formic Acid. For analysis, 20 µL of the sample extract was injected into the LC-MS/MS system. The column was maintained at 50°C. An EMS scan with IDA criteria and precursor scan at 255 and 283 were obtained for each sample, including the SRM 1950 (standard) + Splashmix (internal standard). The scan range of EMS was from 200 Da to 1000 Da, and for the precursor scan, it was from 400 Da to 1000 Da. The mass spectrometer was operated in negative ionization mode. Gradient and instrumental parameters are reported below.

High performance liquid chromatography (HPLC) gradient conditions:

Time (min)	Solvent A: 0.1% FA in 40:60 Water:ACN with 10mM Ammonium Formate	Solvent B: 0.1% FA in 90:10 IPA/ACN with 10mM Ammonium Formate	Flow Rate (ml/min)
	80.0	20.0	0.5
2.00	80.0	20.0	0.5
2.10	60.0	40.0	0.5
10.00	30.0	70.0	0.5
10.10	0.0	100.0	0.5
15.00	0.0	100.0	0.5
15.10	80.0	20.0	0.5

LC-MS/MS Parameter Table

CUR	20.00
CAD	Low
TEM	650.00
GS1	55.00
GS2	60.00
IS	-4500.00
DP	-90.00
EP	-10.00
CE	-25.00

Following data collection, the analysis was carried out using LipidView and the LIPID MAPS® Structure Database (LMSD) software. These platforms enabled the comparison of user data with an extensive external lipid database. Lipid identification was based on precise mass measurements. Initially, the raw data was imported into the software, and m/z values were aligned. Signal intensities underwent normalization using the pooled blank sample and were adjusted to rectify any signal variations encountered during the run (batch correction). Oxidized phospholipids were identified utilizing the SCIEX Analyst software, as referenced (Spiro Khoury et al., 2018), where the precursor mass was manually identified in accordance with the Phosphatidylethanolamine (PE) and Phosphatidylcholine (PC) product ions mass 255 and 283, respectively.

Reference:

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology, 37(8), 911-917.

Spiro Khoury; Corinne Pouyet; Bernard Lyan; Estelle Pujos-Guillot. Evaluation of oxidized phospholipids analysis by LC-MS/MS. Analytical and Bioanalytical Chemistry (2018) 410:633–647.