Glycerophospholipid Identification and Quantitation by Electrospray Ionization Mass Spectrometry

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General Strategy for Phospholipid Isolation and Mass Spectral Analysis

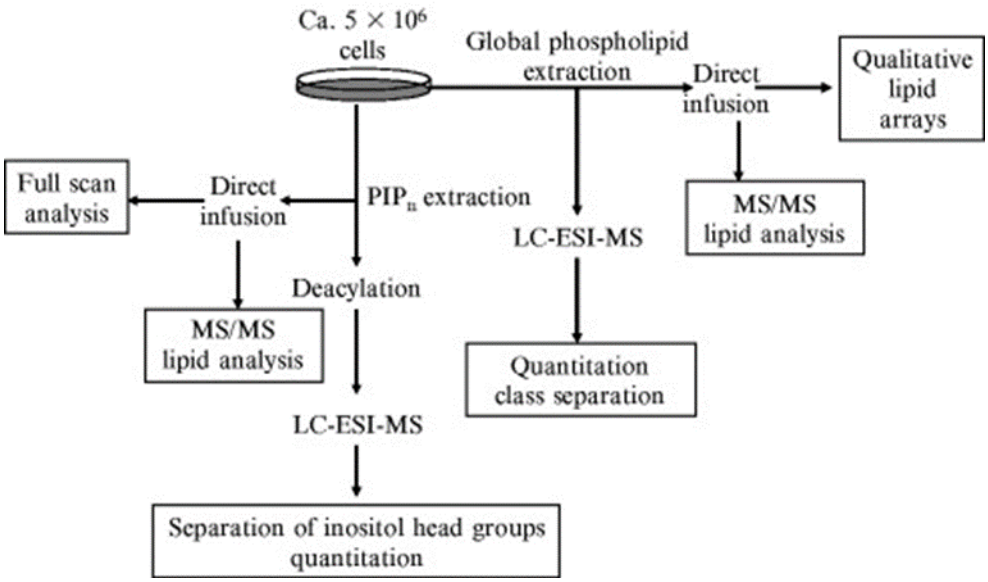


Figure 1. General strategy for the extraction and analysis of glycerophospholipids from cell cultures or tissue.

Phospholipid extraction from cultured cells

As an example of our lipidomics approach for phospholipid analysis by ESI-MS, we use RAW 264.7 cells. Phospholipids were extracted using a modified Bligh and Dyer procedure. The method is suitable for extraction from cell culture plates (100 mm) after aspirating the medium and washing the adhered cells twice with 5 ml of ice-cold 1X phosphate-buffered saline (PBS).

Approximately 1×10^7 cells are then scraped using 800 µl of cold 0.1 N HCl:CH_3OH (1:1) and transferred into cold 1.5-ml microfuge tubes (# L292351, Laboratory Product Sales, Rochester, NY). Other tubes can be used but should be checked with pure solvent prior to usage to ensure that impurities (plastic stabilizers, etc.) are not extracted from the plastic.

After addition of 400 µl of cold $CHCl_3$, the extraction proceeds with vortexing (1 min) and centrifugation (5 min, 4°, 18,000×*g*). The lower organic phase is then isolated and solvent evaporated (Labconco Centrivap Concentrator, Kansas City, MO). The resulting lipid film is rapidly reconstituted in 80 µl CH_3OH:$CHCl_3$ (9:1). Prior to analysis, 1 µl of NH_4OH (18M) is added to each sample to ensure protonation of lipid species.

This method is also appropriate for extraction of phospholipids from previously isolated cell pellets while observing the restrictions for working fast and on ice at all times. After washing the adhered cells (from a 100-mm plate) twice with 5-ml, ice-cold 1X PBS, cells are scraped in 1 ml of 1X PBS, centrifuged (600×*g*, 4°, 5 min), and, after aspirating off the PBS, quickly frozen in liquid nitrogen in the event of transportation or extraction. The extraction of cell pellets follows the same procedure as described above by vortexing the pellet with 800 µl of cold 0.1 N HCl:CH_3OH (1:1) and adding 400 µl of $CHCl_3$ for phospholipid extraction.

Phospholipid extraction from tissue

Samples from tissue biopsies (20 to 50 mg) can also be extracted by this method. In this instance, the samples are quickly frozen by immersion in liquid nitrogen (stored at −80°, if not extracted immediately). The frozen samples are then placed in a tight-fit glass homogenizer (Kimble/Kontes Glass Co, Vineland, NJ), 800 µl of cold 0.1 N HCl:CH_3OH (1:1) is added, and the sample is homogenized for about 1 min while working on ice.

The suspension is transferred to a cold microfuge tube, 400 µl of ice-cold $CHCl_3$ is added, and the extraction proceeds as previously described. Care should be taken in using individual (or cleaned in between) homogenizers to prevent sample cross-contamination.

Direct infusion mass spectrometry of phospholipid extracts

Three major lipid classes can be detected in positive ESI mode: GPCho, GPSer, and GPEtn. Fragmentation of the choline containing GPCho results in a characteristic *m/z* 184 phosphocholine headgroup peak and a $[M+H-59]^+$ peak corresponding to the neutral loss of $(CH_3)_3N$. Relatively small peaks corresponding to the loss of one of the fatty acyl substituents as a ketene $[M-R_2CH=C=O]^+$ (or so-called lyso GPCho) and lyso GPCho-H_2O are also detected. In addition to the diacyl GPCho compounds, a large number of plasmanyl and plasmenyl phosphocholines can also be identified. Over, 100 phosphatidylcholine lipids have been identified in RAW 264.7 cell extracts. GPSer fragmentation in positive mode produces ions resulting from the neutral loss of the polar headgroup phosphoserine ([M+H-185]). Fragmentation of phosphatidyl ethanolamines and lysophosphatidyl ethanolamines in positive mode normally yields one peak, a $[M+H-141]^+$ ion from the neutral loss of the phosphoethanolamine headgroup. Again, plasmanyl and plasmenyl lipids were a large portion of the over 130 GPEtn species identified to date.

Six major lipid classes can be detected in negative ESI mode: GPIns, GPSer, GPGro, GPA, GPEtn, and chloride adducts of GPCho (note that chloride is from decomposition of $CHCl_3$). Additionally, the lyso variants for six of these phospholipids can also be detected in this mode. Negative mode fragmentation of these species yielded a wealth of structural information (**Table 1**). In each case, head group fragmentation, lyso-lipid formation, and fatty acid fragments aided in the lipid identification process.

Phosphatidylinositol fragmentation can generate a wide variety of product ("daughter") ions (Hsu and Turk, 2000c). Four types of lysophosphatidic acid and lysophosphatidylinositols and five characteristic head group fragments can routinely be used in identifying the observed GPIns and lyso GPIns species. In a similar fashion, GPSer and lyso GPSer compounds can be identified from their phosphatidic ($[M-H-Y]^-$) and lyso phosphatidic acid ($[M-H-Y-RCH=C=O]^-$) fragments. The product ion spectra of GPGro also contain an ion formed by the loss of *sn*-2 substituent R_2COOH from its phosphatidic acid ion [M-74]. Fully annotated MS/MSspectra for examples from all six major glycerophospholipid classes are available on the LIPID MAPS public website : http://lipidmaps-dev.babraham.ac.uk/resources/standards.php

Table 1. Negative product ("daughter") ions routinely observed from fragmentation of glycerophospholipids

	GPA	GPCho (Cl)	GPEtn	GPGro	GPIns	GPSer
$[M-H]^-$	X		X	X	X	X
$[M-H-RCO\equiv C\equiv O]^-$	X		X	X	X	
$[M-H-RCOOH]^-$	X		X	X	X	
$[M-H-RCOOH]^-$				X	X	X
$[M-H-headgroup]^-$			X		X	X
$[M-H-headgroup-RCH\equiv C\equiv O]^-$			X	X	X	X
$[M-H-headgroup-RCOOH]^-$			X		X	
$[M-H-RCHCO]^-$			X	X	X	
$[M-H-RCHCO-2H_2O]^-$				X	X	
$[RCOO]^-$	X	X	X	X	X	X
Head-group–specific ion	135		140, 196	171	241, 223	
$[GP-H_2O-H]^-$ (153)	X	X	X	X	X	X
$[H_2PO_4]^-$	X	X	X	X	X	X
$[PO_3]^-$	X	X	X	X	X	X
$[M+35]^-$ $[M+3]^-$ (M+Cl)		X				
[M+CL–CH3]− (M–50) (M–52)		X				

Mass spectral analysis was performed on a Finnigan TSQ Quantum triple quadrupole mass spectrometer (Thermo Finnigan, San Jose, CA) equipped with a Harvard Apparatus syringe pump (Harvard Apparatus, Holliston, MA) and an electrospray source. Samples were analysed at an infusion rate of 10 µl/min in both positive and negative ionization modes over the range of *m/z* 400 to 1200. Instrument parameters were optimized with 1, 2-dioctanoyl-*sn*-Glycero-3-phosphoethanolamine (16:0 GPEtn) prior to analysis. Examples of full-scan spectra in negative and positive instrument modes are shown in **Fig. 2**. Data were collected with the Xcalibur software package (Thermo Finnigan) and analysed by a software program developed in our research group (see "Computational Analysis of Mass Spectral Data" section for a detailed description of data analysis).

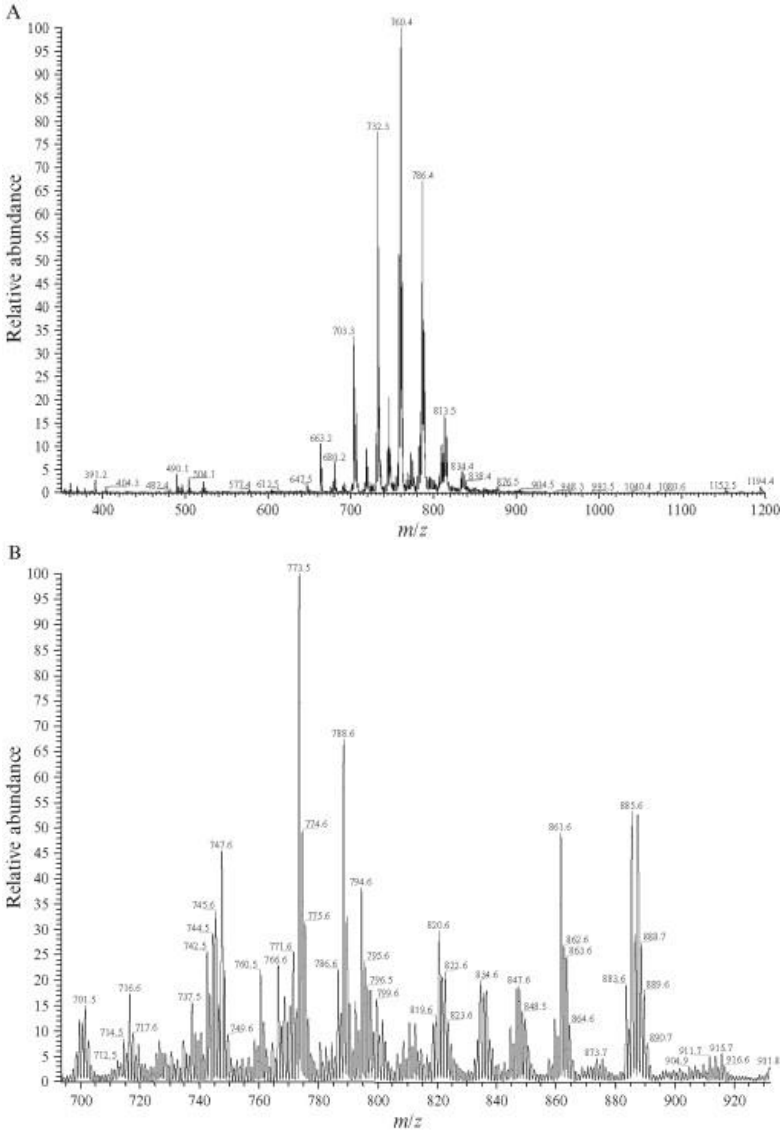


Figure 2. Examples of glycerophospholipid direct infusion spectra. (A) Positive instrument mode spectra showing GPCho, GPEtn, and GPSer lipids. (B) Negative instrument mode showing GPA, GPCho (Cl adducts), GPEtn, GPGro, GPIns, and GPSer lipid species.

LC-MS analysis (quantitation) of phospholipid extracts

Quantification of glycerophospholipids is achieved by the use of a LC-MS technique employing synthetic (non-naturally occurring) diacyl (**Table 3**) and lysophospholipid standards (**Table 4**).
Typically, 200 ng of each odd-carbon standard is added per 10^7 cells. The extraction of lipids from cell culture or tissue biopsies is the same as described previously for the direct infusion MS analysis (see "Extraction of Glycerophospholipids" section). After solvent evaporation, the resulting lipid film is dissolved in 100 µl of Isopropanol(IPA):Hexane:100 m*M* $NH_4CO_2H_{(aq)}$ 58:40:2 (mobile phase A).

For our examples, we utilized an Applied Biosystems/MDS SCIEX 4000 Q TRAP hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA).
Coupled to this instrument were a Shimadzu (Shimadzu Scientific Instruments, Inc., Columbia, MD) HPLC system consisting of a SCL 10 AVP controller, two LC 10 ADVP pumps and a CTC HTC PAL autosampler (Leap Technologies, Carrboro, NC). All samples were separated on a Phenomenex (Phenomenex, Torrance, CA) Luna Silica column (2 × 250 mm, 5-µ particle size) using a 20-µl sample injection.
Lipids were separated using a binary gradient program consisting of IPA:Hexane:100 m*M* $NH_4CO_2H_{(aq)}$ 58:40:2 (mobile phase A) and IPA:Hexane:100 m*M* $NH_4CO_2H_{(aq)}$ 50:40:10 (mobile phase B). The following LC gradient was used: 0 to 5 min, B = 50%; 5 to 30 min, B = 50 to 100%; 30 to 40 min, B = 100%; 40 to 41 min, B = 100 to 50%; and 41 to 50 min, B = 50%.
The mobile phase was delivered at a flow rate of 0.3 ml/min. The MS spectra were acquired in negative instrument mode using a turbo spray source operated at 450° with an ion voltage of −3500 V, and nitrogen as curtain and nebulizer gas. The curtain gas (CUR) was 30 l/hr, and ion source gas 1 and 2 were both 50 l/hr.
The declustering potential (DP) was −110 V, and the collision energy (CE) was −5 V. Scan type: EMS, unit resolution for Q1; scan rate: 1000 amu/s; scan range from *m/z* 350 to 1200, with the ion trap set for dynamic fill time. As an example of this technique, extracted ion chromatograms (XICs) of the four GPGro odd-carbon standards in a RAW 264.7 cell background are shown in **Fig. 3**. The creation of standard curves and quantitation of data will be described in the "LC-MS Data Analysis" section.

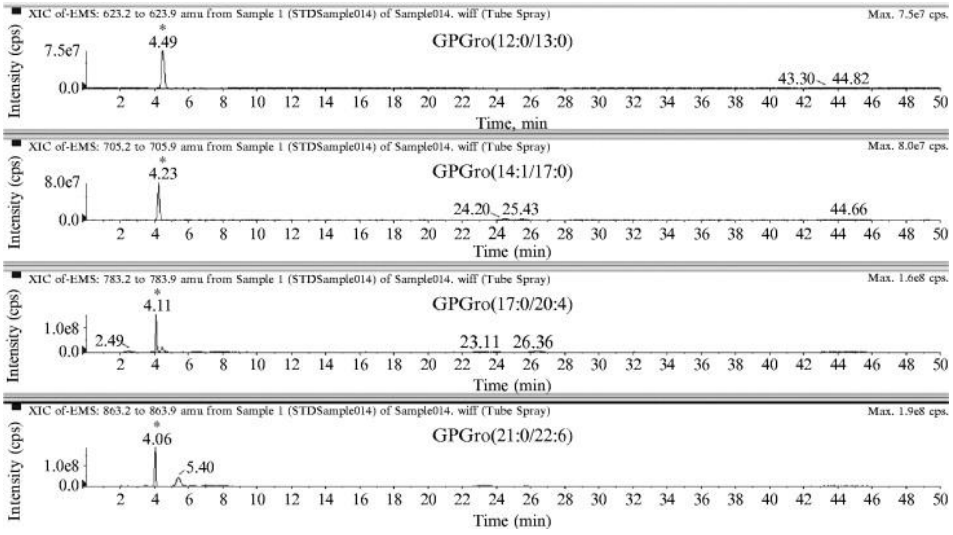
Table 3. Odd-carbon standards for liquid chromatography-mass spectometry analysis of phospholipids: Twenty-four odd-carbon diacyl standards

	12:0/13:0	**17:0/14:1(9Z)**	**17:0/20:4 (5Z,8Z,11Z,14Z)**	**21:0/22:6 (4Z,7Z,10Z,13Z,16Z,19Z)**
GPA	25:00:00	31:01:00	37:04:00	43:06:00
GPCho	25:00:00	31:01:00	37:04:00	43:06:00
GPEtn	25:00:00	31:01:00	37:04:00	43:06:00
GPGro	25:00:00	31:01:00	37:04:00	43:06:00
GPIns	25:00:00	31:01:00	37:04:00	43:06:00
GPSer	25:00:00	31:01:00	37:04:00	43:06:00

Table 4. Odd-carbon standards for liquid chromatography-mass spectrometry analysis of phospholipids: Four lysolipid standards

13:0/0:0	**17:1(9Z)/0:0**
13:0 LysoGPA	17:1 LysoGPA
13:0 LysoGPCho	17:1 LysoGPCho

Figure 3. Extracted ion chromatograms of the four GPGro odd-carbon internal standards



Polyphosphoinositide Extraction and Mass Spectral Analysis

Polyphosphoinositides are low-abundance phospholipids in eukaryotic cell membranes that are involved in regulation of distinct cellular processes. In order to minimize the interference of other phospholipids during analysis, a selective two-step extraction is employed. First, the cell material is extracted with neutral solvents and the resulting pellet is extracted with acidified solvents for quantitative recovery of polyphosphoinositides. The majority of non-inositol phospholipids are extracted with neutral solvents, and no GPInsP_2 or GPInsP_3 species are detected in this extract. The acyl composition of polyphosphoinositides is determined by direct infusion and fragmentation MS. The quantification and analysis of headgroup regioisomers is accomplished by LC-MS analysis after deacylation.

Extraction of polyphosphoinositides from cultured cells

Cells for polyphosphoinositide analysis must be between 60 to 70% confluency at the time of harvesting, unless the experiment specifically requires otherwise. Systematic effects of confluence have been previously observed in our laboratory. Cells from 100 to 150 mm culture plates are washed with 2 ml of ice-cold 1X PBS after aspirating the culture medium. Then, 1.5 ml of 1X PBS is added, and cells are scraped and transferred into a cold 1.5-ml microfuge tube. Cell pellets are collected after centrifugation at 4000×*g* for 5 min at 4°.

The procedures are performed on ice, and all solutions and sample tubes are kept on ice at all times. Each pellet is given 400 µl of ice-cold 1:1 $CHCl_3$:CH_3OH and vortexed for 1 min, or until thoroughly mixed. Samples are centrifuged at 7500×*g* for 5 min at 4°, supernatant decanted, and discarded.

To the remainder of the cell pellet, 200 µl of 2:1 $CHCl_3$:CH_3OH containing 0.25% 12 N HCl are added. Samples are vortexed for 5 min and then pulse spun. The supernatant is then given 40 µl of 1 N HCl and vortexed for 15 s. The resulting two phases are separated by centrifugation (pulse at 18,000×*g*). The solvent from the collected lower layer is evaporated in a vacuum centrifuge (Labconco CentriVap Concentrator, Kansas City, MO). The resulting lipid film is rapidly re-dissolved in 55 µl of 1:1:0.3 $CHCl_3$:CH_3OH:H_2O. Before analysis, 5 µl of 300 m*M* piperidine is added as an ionization enhancer, and the sample is vortexed and pulse spun.

Extraction of polyphosphoinositides from tissue

Biopsy samples (20 to 50 mg) are quickly frozen by immersion in liquid nitrogen. Samples can be stored at −80°, if not immediately extracted. The frozen samples are homogenized using 500 µl of CH_3OH in a Dounce tissue grinder (Kimble/Kontes Co., Vineland, NJ) for about 1 min working on ice.

The suspension is then transferred to a cold 1.5-ml microfuge tube, and 500 µl of cold $CHCl_3$ are added. After vortexing for 1 min at 4°, samples are centrifuged at 7500×*g* for 5 min at 4°. Supernatant is decanted and discarded. The retained pellet is then given 200 µl of 2:1 $CHCl_3$:CH_3OH containing 0.25% 12 N HCl.

Samples are vortexed for 5 min and then pulse spun. Supernatant is transferred to a new cold microfuge tube and, after addition of 40 µl of 1 N HCl, the samples are vortexed for 15 s. The resulting two phases are separated by centrifugation (pulse at 18,000×*g*). The solvent from the collected lower layer is evaporated in a vacuum centrifuge. The resulting lipid film is rapidly re-dissolved in 55 µl of 1:1:0.3 $CHCl_3$:CH_3OH:H_2O.

Before analysis, 5 µl of 300-m*M*piperidine is added as an ionization aid, and the sample is vortexed and pulse spun.

Direct-infusion mass spectral analysis of polyphosphoinositides

Mass spectral analysis was performed on an Applied Biosystems/MDS SCIEX 4000 Q TRAP hybrid, triple-quadrupole, linear ion trap MS (Applied Biosystems, Foster City, CA).

The instrument was equipped with a Harvard Apparatus syringe pump and an ESI source. Samples were analysed at an infusion rate of 10 µl/min in negative ionization mode over the range of *m/z* 400 to 1200. GPIns*P* and GPInsP_2 were analysed by full scan analysis and normalized to 16:0 GPInsP_2 internal standard (Avanti Polar Lipids, Alabaster, AL).

Utilizing this method, approximately 30 GPIns*P* and GPInsP_2 species can be detected. A typical RAW 264.7 full-scan spectra highlighting the GPIns*P* and GPInsP_2spectral region is shown in **Figure 4**.

Due to their very low abundance and detection only after stimulation, GPInsP_3 species cannot be identified by full or precursor ion scan (*m/z* 481). Instead, identification of the individual phosphatidylinositol

phosphates present in the total lipid extracts was accomplished by ESI-MS/MS with a collision energy of 50 eV. Peaks corresponding to known GPInsP_3 compounds were fragmented and manually inspected for the presence of the identification peaks (examples of GPIns$P$, GPInsP_2, and GPInsP_3 fragmentation patterns can be found on the LIPID MAPS public website: http://lipidmaps-dev.babraham.ac.uk/resources/standards.php

A confirmed identification was achieved when key fragmentation peaks were larger than three times the signal-to-noise ratio (S/N). The limit of detection using this method was found to be less than 9 pmol/ml for 38:4 GPInsP_3 (Avanti Polar Lipids, Alabaster, AL). Data were collected with the Analyst software package (Applied Biosystems).

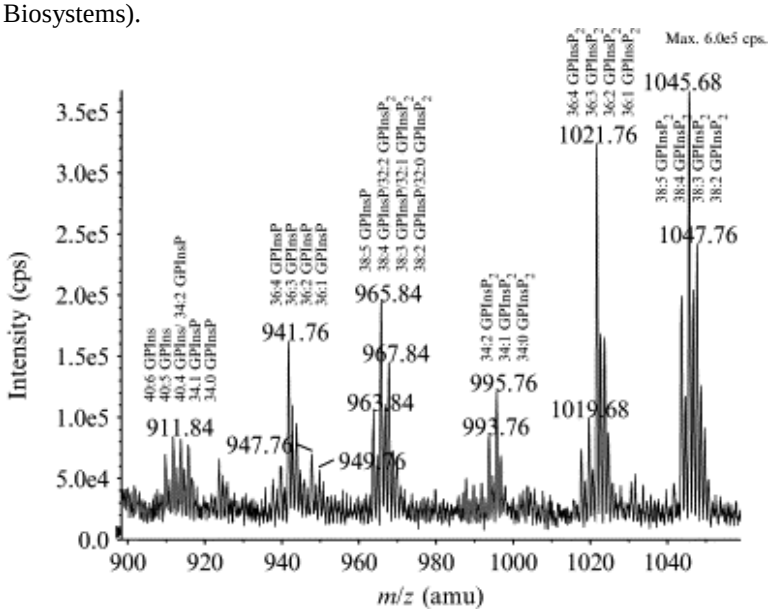


Figure 4. GPIns$P$ and GPInsP_2 spectral region of a RAW 264.7 cell extract. Approximately 30 chemically distinct GPIns$P$ and GPInsP_2 species can be identified by tandem mass spectrometry fragmentation in a typical spectrum.

Deacylation of GPInsPn lipids

The quantitative analysis of headgroup isomeric GPInsP_2 and GPInsP_3 is achieved by LC-MS analysis of the deacylated polyphosphoinositides and the use of cytidine-5′-monophosphate (CMP; Sigma-Aldrich, St. Louis,MO) as an internal standard.

The lower layer of the last step of the extraction protocol that yields separation of phosphoinositides from the rest of the phospholipids is subjected to deacylation as described by other authors.

The organic phase (lower layer) is transferred into a glass screw-cap vial and solvent-evaporated under nitrogen. Afterward, 500 µl of freshly prepared methylamine reagent (1-butanol, methanol, 40% aq. methylamine, 1:4:4, v/v) was added to the glass vial and heated at 53° in a heating block (water bath) for 1 hr. After cooling to room temperature, the content is transferred into a microfuge tube and solvent-evaporated in a vacuum centrifuge (Labconco CentriVap Concentrator, Kansas City, MO).

The resulting film is resuspended in 500 µl of distilled H_2O, vortexed briefly, and centrifugated for 2 min at 18,000×g. Supernatant is transferred to a new microfuge tube. GroPInsP_n are extracted with 500 µl of 1-butanol/petroleum ether/ethyl formate (20:4:1,v/v) to remove the undeacylated lipids and free fatty acids.

After mixing (by vortex) for 30 s and centrifugation (18,000×g, 2 min), the lower (aqueous) layer is collected. The extraction is repeated, and the upper phase is discarded. Combined aqueous phases are dried in a vacuum centrifuge (or lyophilized). The samples are resuspended in 36 µl of methanol:water (1:1), mixed with 40 µl of 100-µM CMP and 4 µl of 300-mM piperidine, and analysed by LC-MS.

The phosphoinositides standards were deacylated likewise with methylamine and dissolved in methanol:water (1:1) containing 10% 300-mM piperidine to produce the stock solution with concentration of 200 µM. The stock solution was further serially diluted to obtain working solution over the range 20 to 200 µM.

LC-MS analysis of deacylated GPInsPn compounds

Here we describe an LC-MS method for separation, identification and quantification of the four GroPInsP_2 and GroPInsP_3 lipid species from cell extracts by using β-cyclodextrin–bonded column with on-line negative ESI-MS detection and internal standard calibration.

The HPLC system consisted of two Shimadzu (Shimadzu Scientific Instruments, Inc., Columbia, MD) LC-10 ADVP binary pumps and an SCL-10AVP system controller. A 20-µl sample was injected via a CTC HTC PAL autosampler (Leap Technologies, Carrboro, NC). The separation was performed on a Nucleodex β-OH column, 5 µm (200 × 4.0 mm i.d.) from Macherey-Nagel (Düren, Germany). The mobile phase included 50-m*M* ammonium formate aqueous solution (solvent A) and acetonitrile (solvent B). The following gradient was used: 0 to 1 min, B = 80%; 1 to 15 min, B = 80 to 70%; 15 to 30 min, B = 70 to 66%; 30 to 70 min, B = 66 to 61%; 70 to 75 min, B = 61 to 60%; 75 to 76 min, B = 60 to 80%; and 76 to 90, B = 80%. The mobile phase was delivered at a flow rate of 0.3 ml/min.
The MS spectrum was acquired in negative mode on a 4000 Q-Trap LC-MS/MS system, fitted with turbo spray ion source from Applied Biosystems (Foster City, CA). The turbo spray source was operated at 200° with an ion voltage of −4500 V, and nitrogen as CUR and nebulizer gas. The CUR was 10 l/hr. The flow rate of ion source gas 1 and gas 2 was 20 and 40 l/hr, respectively. The declustering potential (DP) was −110 V, and the collision energy (CE) was −5 V. Settings include channel electron multiplier (CEM): 2200 V; scan type: enhanced MS (EMS), unit resolution for Q1; scan rate: 1000 amu/s; scan range from *m/z* 300 to 590; duration: 90 min; step size: 0.08 amu; Q3 entry barrier: 8 V; and pause between mass ranges: 5.007 ms. A representative extracted ion current (XIC) is shown on **Fig. 5** depicting the separation of the three GroPInsP_2 and GroPInsP_3 isomers.

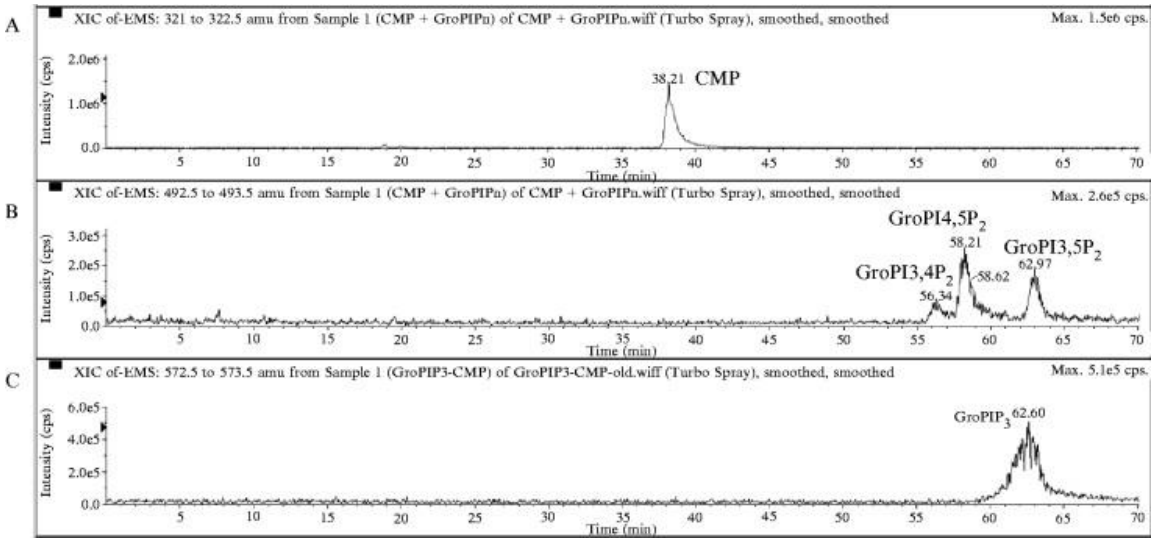


Figure 5. Liquid chromatography-mass spectrometry spectra of GroPInsP_2 and GroPInsP_3 standards with internal standard cytidine-5′-monophosphate (CMP). (A) Extracted ion chromatograms (XICs) of cytidine-5′-monophosphate (*m/z* 322); (B) Extracted ion chromatograms of GroPInsP_2 (*m/z* 493). (C) Extracted ion chromatograms of GroPInsP_3 (*m/z* 573).

Calibration curves were constructed by mixing deacylated GPInsP_n standards working solutions with equal volume of 100-µ*M* CMP. They were then analysed by LC-MS. Calibration curves for deacylated GPIns4,5P_2 and GPIns3,5P_2 are shown on **Fig. 6A** and **B**. They are created by using the relationship between "peak area of GroPIns*Pn*/peak area of CMP" and "amount of GroPIns*Pn*/amount of CMP." As an example, the method was applied to the analysis of RAW 264.7 cell extracts after stimulation with platelet activating factor (PAF) for 15 min. The extracts were deacylated and analysed by LC-MS using CMP as an internal standard. The XIC traces from this analysis are shown on **Fig. 7A** and **B**.

Computational Analysis of Mass Spectral Data

Complex mixtures of phospholipids from biological extracts generate mass spectra with hundreds to thousands of peaks associated with the molecular ionspresent in the sample, which includes any molecules that acquire a charge in the desired instrument mode over the applicable m/z range. In lipid extracts, these ions include phospholipids, solvent contaminants, fragments of larger macromolecules, and multiply charged ions, among other components. In addition, in the absence of chromatographic separation, any particular m/z peak may contain ions from several different isobaric phospholipids or isotopic ions from molecular ions at m/z −1, m/z −2, which may be appropriately corrected for isotopic distribution if the molecular composition is known.

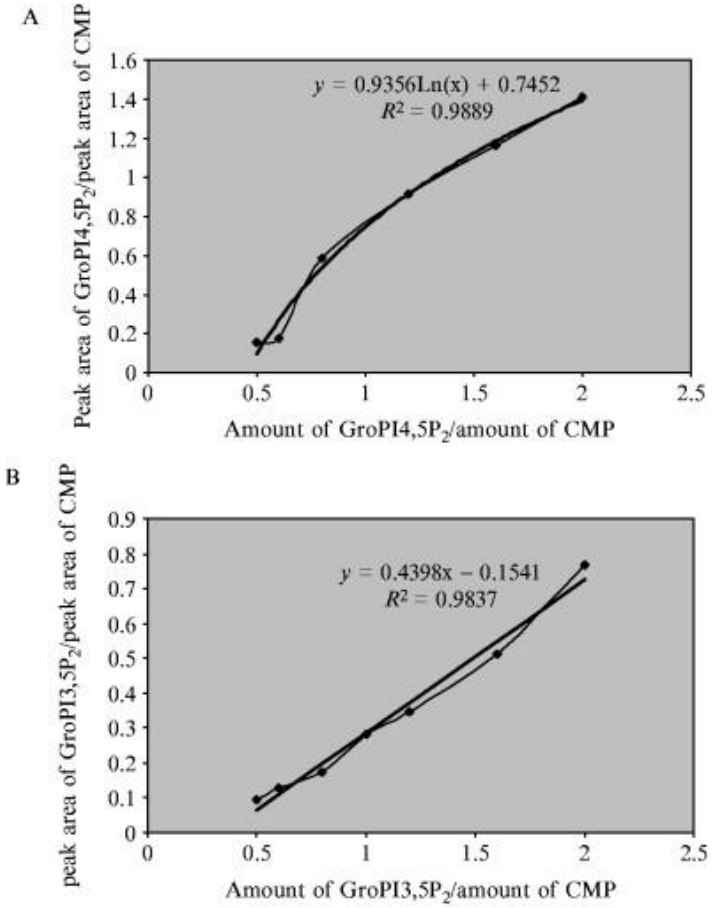


Figure 6. Calibration curves for (A) GroPIns4,5P_2 and (B) GroPIns3,5P_2.

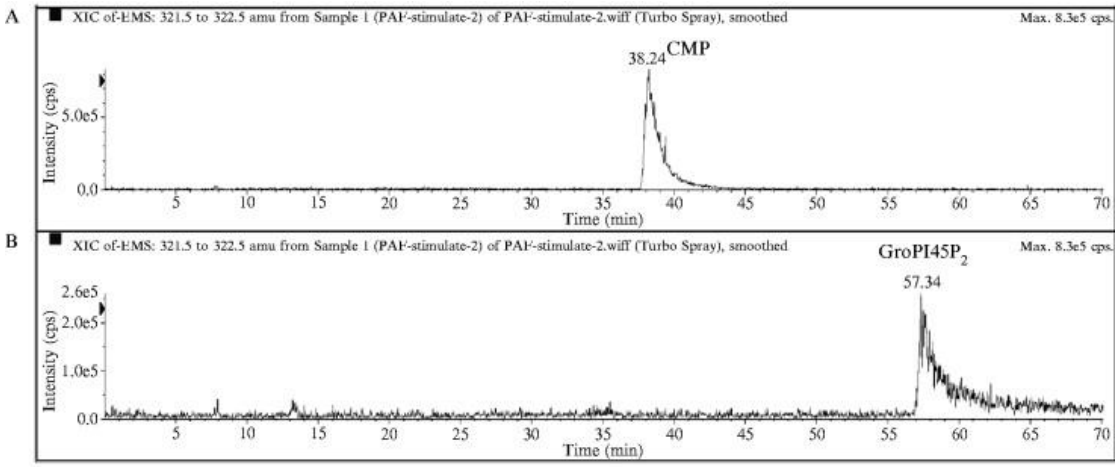


Figure 7. Extracted ion chromatograms (XIC) of GroPIns*Pn* from platelet activating factor (PAF)-stimulated RAW 264.7 cell extracts. (A) Extracted ion chromatograms of cytidine-5′-monophosphate (CMP) (*m/z* 322). (B) Extracted ion chromatograms of GroPInsP_2 (*m/z* 493).

Direct infusion (intra-source separation)

A single typical direct infusion spectrum is binned in 0.07 *m/z* units and spans a range of *m/z* 350 to 1200 (12,142 bins). Each sample is scanned once per second for approximately 60 s (user-defined) so that a single spectrum actually consists of 60 scans. The vendor-supplied software typically time-averages these scans for graphical display. The ASCII data for each MS file can be obtained from the vendor files (.Raw files) by using XConvert.exe

located in the \bin folder of Xcalibur. This program allows batch processing of a large number of .Raw files. The format of these ASCII files (~60 MB) is cumbersome and contains mostly redundant text so that it is useful to write a parsing program to store the relevant time-*m/z*-ion intensity information in separate files. We wrote a program in Fortran (G77) to batch process large numbers of these ASCII files. Details on the program steps appear in the original paper: https://doi.org/10.1016/S0076-6879(07)32002-8.

LC-MS data analysis

The batch processing of full-scan LC-MS data requires significantly more computational processing than the direct infusion data, primarily due to the (typically) 60 min runs per sample, with approximately one scan per second. The MS data are contained in the vendor (Analyst, Applied Biosystems) .wiff files and must be converted into ASCII prior to further processing. We use a converter mzStar.exe from the Institute for Systems Biology (ISB), which converts the .wiff files into mzXML format. We convert the mzXML files to .mgf (Mascot Generic Format, ASCII files) and header files (ASCII) using mzXML2other.exe (ISB), which requires compilation on a cygwin, X-Windows terminal emulating a Unix shell. Each step is performed per batch (processes multiple files without user input), and the .mgf sample files are approximately 100 MB ASCII each. The .mgf files contain sequentially ordered scans (the retention time of each scan is located in the header file) and the *m/z* bins and their associated ion intensity values. We wrote code in Fortran (G77) to batch process these .mgf files creating two forms of output: (1) smoothed (time-averaged in ~10-s increments) XICs of retention time/intensity values for every nominal *m/z* value, and (2) integrated, aligned (by *m/z* and retention time) peaks across the samples. Details on the program steps appear in the original paper: https://doi.org/10.1016/S0076-6879(07)32002-8

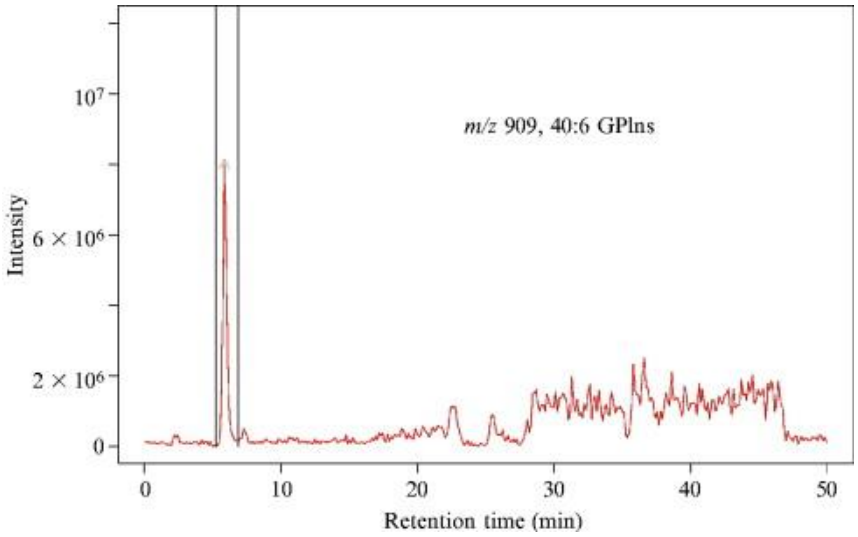


Figure 9. Generation of extracted ion chromatograms (XIC; *m/z* 909.06 to 909.96) from Fortran code as described in the text. The horizontal axis is retention time, and the vertical axis ion intensity. The vertical black lines indicate the region of integration dynamically selected by the code. The red triangle indicates the peak(s) automatically identified by the algorithms described in the text.

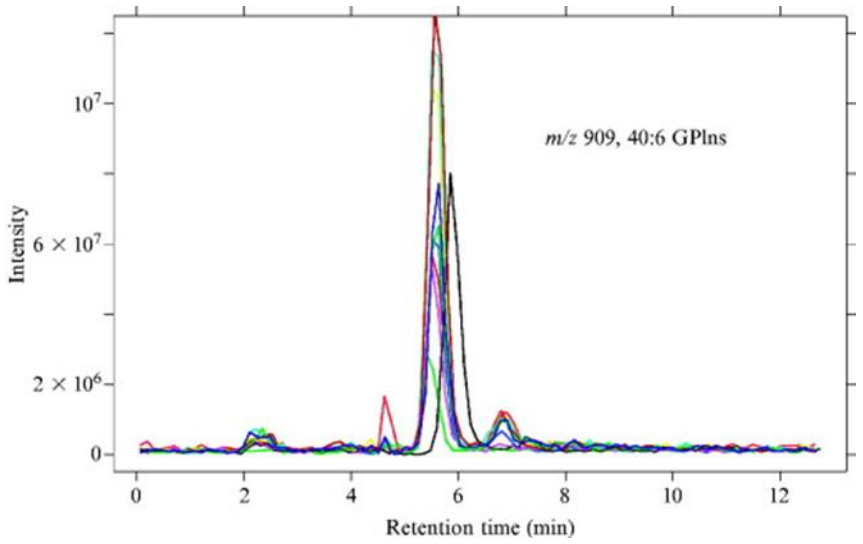


Figure 10. A zoomed-in section including the extracted ion chromatogram (XIC) from **Fig. 9** (*m/z* 909.06 to 909.96), which also includes extracted ion chromatograms generated from nine other full-scan spectra files, indicative of the variation in amplitude and peak location across these files. Peak areas from the 10 files are automatically aligned as described and output in ASCII format for further analysis (normalization and quantification). Many mass-to-charge (*m/z*) values in glycerophospholipid (GPL) full-scan spectra contain extracted ion chromatograms with multiple peaks at different retention times.

Following the generation of integrated, aligned peak areas across multiple spectra files, accurate quantification of molecular species requires titrations of internal standards for individual species of phospholipids over the presumed physiological range of variation in abundance. To generate standard curves, we proceed as follows: (1) add fixed quantities of multiple fixed odd-chain internal standards (per glycerophospholipid [GPL] class) to every sample; (2) add varying amounts of even-carbon standards (e.g., 10, 50, 100, 500 ng) in triplicate; (3) normalize the peak areas of the even-carbon standards to the average of the fixed odd-carbon areas (per GPL class); and (4) repeat the process several times to estimate experiment to experiment variability in the slopes and intercepts (assuming linearity). An example of standard curves for the GPA class using experimental protocol and code described above is given **in Fig. 11**, and the variation in slope values and intercepts across independent experiments for the even-carbon internal standards are listed in **Table 5**.

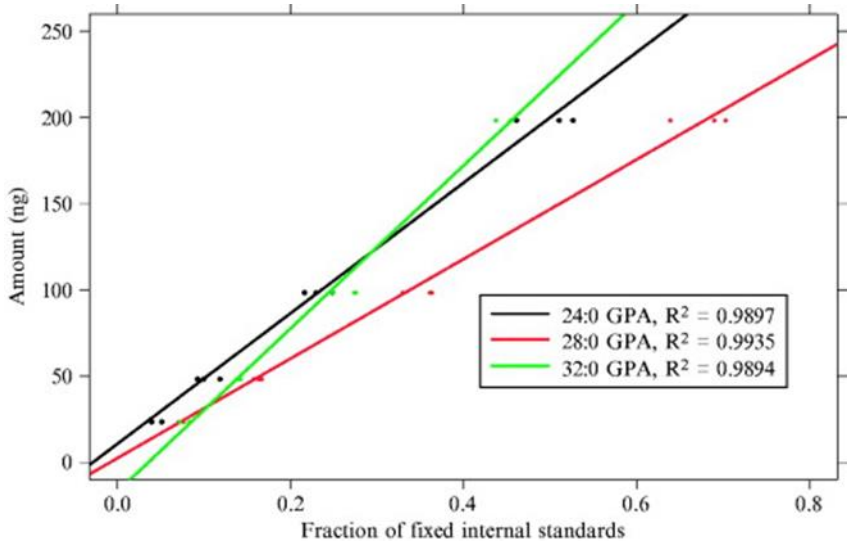


Figure 11. Three standard curves for 24:0, 28:0, and 32:0 glycerophosphatidic acid (GPA) (black, green, and red, respectively) generated as described in the text using the automated code. The horizontal axis refers to peak areas normalized to the mean of four fixed odd-carbon internal standards, and the vertical axis is the amount added in nanograms (ng).

Table 5. Variation in glycerosphosphatidic acid (GPA) slopes across independent experiments

	Experiment		
GPA	***1*** [a]	***2*** [a]	***3*** [a]
24:00:00	254	289	X
28:00:00	189	184	X
32:00:00	238	267	266
34:01:00	172	244	204
34:02:00	147	179	145
36:00:00	192	352	330
36:01:00	206	247	202
36:02:00	195	288	194
38:04:00	253	218	230
38:06:00	299	388	337
40:06:00	437	403	384

a Standard deviations in slope values for individual experiments are approximately 5%.

For LC-MS with the protocol as described, we have found that the variation in slopes across GPLs within a class is nonlinear (and class-dependent) and has no obvious dependence on double bonds or carbon number. This is unfortunate since only rough slope and intercept estimates are possible for molecular species for which internal standards are not available. These estimated slopes for molecular species that lack titrations are based on the variation in slopes for other members of the same GPL class. One may use the range of slopes or regression estimates (e.g., in *m/z*) across the GPL class to estimate the unknown slopes with appropriately large errors in the quantification of these molecular species. In **Table 6**, we list estimated amounts of different GPSer in RAW 264.7 cells following the LC-MS protocol described in the previous section. Once the molecular identity at a nominal *m/z* and retention time is known, isotopic corrections for *m/z* +2 contributions from the molecular species at a nominal *m/z* are readily computed and normalized peak areas may be appropriately corrected and quantified within a GPL class. External normalization (e.g., cell counts, DNA) is useful for comparing across samples as the fluctuation in individual GPLs may be significant due to extrinsic factors. Addition of internal standards prior to extraction may be used to estimate extraction efficiency per class and potential differential extraction efficiencies within a GPL class.

Table 6. Approximate amounts (ng) of individual GPSer molecular species in RAW 264.7 cells (5×10^6 cells)

GPSer[a]	**Estimated amount (ng)**		**GPSera**	**Estimated amount (ng)**	
32:01:00	52	40	**38:04:00**	188	326
32:00:00	30	24	**38:03:00**	293	340
34:03:00	41	18	**38:02:00**	144	12
34:02:00	62	54	**38:01:00**	60	65
34:01:00	393	441	**38:00:00**	9	26
34:00:00	163	172	**40:07:00**	37	47
36:04:00	20	23	**40:06:00**	575	692
36:03:00	30	30	**40:05:00**	770	895
36:02:00	249	314	**40:04:00**	451	538
36:01:00	1119	1319	**40:03:00**	200	260
36:00:00	464	510	**40:02:00**	78	87
38:06:00	21	25	**40:00:00**	33	41
38:05:00	60	70			

a Shown are duplicate control (unstimulated) samples for a 2-h KDO2 Lipid A stimulation using a 10% serum LIPID MAPS protocol (www.lipidmaps.org).