

Analysis of intact phosphoinositides in biological samples

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Abstract It is now apparent that each of the known, naturally occurring polyphosphoinositides, the phosphatidylinositol monophosphates (PtdIns3P, PtdIns4P, PtdIns5P), phosphatidylinositol bisphosphates [PtdIns(3,4)P₂, PtdIns(3,5)P₂, PtdIns(4,5)P₂], and phosphatidylinositol trisphosphate [PtdIns(3,4,5)P₃], have distinct roles in regulating many cellular events, including intracellular signaling, migration, and vesicular trafficking. Traditional identification techniques require [³²P]inorganic phosphate or [³H]inositol radiolabeling, acidified lipid extraction, deacylation, and ion-exchange head group separation, which are time-consuming and not suitable for samples in which radiolabeling is impractical, thus greatly restricting the study of these lipids in many physiologically relevant systems. Thus, we have developed a novel, high-efficiency, buffered citrate extraction methodology to minimize acid-induced phosphoinositide degradation, together with a high-sensitivity liquid chromatography-mass spectrometry (LC-MS) protocol using an acetonitrile-chloroform-methanol-water-ethylamine gradient with a microbore silica column that enables the identification and quantification of all phosphoinositides in a sample. The liquid chromatograph is sufficient to resolve PtdInsP₃ and PtdInsP₂ regioisomers; however, the PtdInsP regioisomers require a combination of LC and diagnostic fragmentation to MS³. Data are presented using this approach for the analysis of phosphoinositides in human platelet and yeast samples.—Pettitt, T. R., S. K. Dove, A. Lubben, S. D. J. Calaminus, and M. J. O. Wakelam. **Analysis of intact phosphoinositides in biological samples.** *J. Lipid Res.* 2006. 47: 1588–1596.

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As our understanding of lipids as highly dynamic structures with both structural and signaling roles matures, one group stands out. These are the phosphoinositides, which regulate a host of cellular events, such as membrane trafficking, secretion, adhesion, migration, cell survival, and replication (1–4). Many of these outcomes are regulated by interactions between phosphoinositides and effector

proteins bearing specific phosphoinositide binding domains (e.g., ENTH, FYVE, PH, PX) (5). Within this lipid family, the best known are phosphatidylinositol (PtdIns), the parent of the higher phosphorylated forms, which itself has no known signaling role, phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P₂], and phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P₃]. PtdIns(4,5)P₂ is the precursor to diacylglycerol and inositol 1,4,5-trisphosphate in receptor signaling and also directly regulates cytoskeletal reorganizations and the activities of enzymes such as phospholipase D. PtdIns(3,4,5)P₃ regulates cell movement, apoptosis, metabolism, and proliferation via the activation of phosphoinositide dependent kinase 1, protein kinase B, and other PH and PX domain proteins. However, it is now also apparent that other PtdInsP₂ regioisomers, such as phosphatidylinositol 3,4-bisphosphate [PtdIns(3,4)P₂] and phosphatidylinositol 3,5-bisphosphate [PtdIns(3,5)P₂], together with the phosphatidylinositol monophosphate regioisomers phosphatidylinositol 3-phosphate (PtdIns3P), phosphatidylinositol 4-phosphate (PtdIns4P), and phosphatidylinositol 5-phosphate (PtdIns5P), have their own specific functions. PtdIns3P and PtdIns4P regulate membrane trafficking, whereas PtdIns5P has been reported to bind to the PHD domain in the candidate tumor suppressor nuclear protein ING2 (6). PtdIns(3,4)P₂ binds PH and PX domains in many proteins, whereas PtdIns(3,5)P₂ can bind to ENTH and a subset of WD40 domains involved in autophagy and stress responses (7). Mutations in the signaling pathways involving generation, removal, or responses to these lipids have been detected in many cancers and other diseases, further emphasizing the critical functions of the phosphoinositides (8–11).

To fully understand the role of these phosphoinositides, we need the ability to follow their individual changes. As it becomes clear that the different regioisomers have

Abbreviations: LC-MS, liquid chromatography-mass spectrometry; PtdIns, phosphatidylinositol; PtdInsP, phosphatidylinositol monophosphate; PtdInsP₂, phosphatidylinositol bisphosphate; PtdInsP₃, phosphatidylinositol 3,4,5-trisphosphate.

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different signaling functions, exact isomeric identification is essential. At present, this is very difficult, requiring equilibrium [^3H]inositol or [^{32}P]inorganic phosphate radiolabeling of cells before extraction. Equilibrium radiolabeling is not possible for many primary cells, as they cannot be stably maintained in culture for a sufficient length of time. After radiolabeling, the lipids are extracted and deacylated to remove fatty acids, and the glycerophosphoinositol head groups are resolved by ion-exchange chromatography. Because most of these structures are normally only found in trace amounts [e.g., PtdIns(3,4,5) P_3 in resting cells is present at only $\sim 0.1\%$ of its precursor PtdIns(4,5) P_2 , although it can increase up to 100-fold after stimulation], the multiple manipulation steps required often result in substantial losses, making these techniques inappropriate for many studies in which starting material is limited. Deacylation also loses all structural information relating to the acyl part of the molecule, which may prove important in the same way that the mammalian signaling forms of diacylglycerol have polyunsaturated acyl compositions and signaling phosphatidic acid normally has monounsaturated and diunsaturated acyl compositions (12). Indeed, recent work suggests that the PtdIns(3,4,5) P_3 acyl structures formed may be stimulus-dependent (13).

Thus, there is an urgent need to develop novel, sensitive methods to detect and resolve intact phosphoinositides. A few reports of tandem mass spectrometry identification of PtdIns P , PtdIns P_2 , and PtdIns P_3 have been published (13–16); however, these methods were unable to resolve the regioisomers. Furthermore, these approaches suffer from the ion suppression effects inherent to direct infusion of unfractionated, complex lipid mixtures, which can lead to a significant loss of sensitivity. This is particularly the case for minor components such as the polyphosphoinositides. Here, we report the development of liquid chromatography-mass spectrometry (LC-MS n) procedures that enable the complete identification, at high sensitivity, of all of the phosphoinositides in a complex total lipid extract, without prior fractionation, and apply the techniques to analyze physiologically relevant biological samples.

MATERIALS AND METHODS

Platelet isolation

Human venous blood was drawn by venipuncture from healthy volunteers into sodium citrate and acid-citrate-dextrose as described previously (17). Washed platelets were isolated and used at 2×10^9 /ml. Aliquots (0.8 ml) were stimulated in 15 ml polypropylene tubes (Sarstedt) with 1 unit of thrombin per milliliter at 37°C.

Yeast growth and treatment

Yeast strain BY4742 was grown in synthetic complete medium to a density of 1×10^7 cells/ml and treated with either medium or medium containing 0.9 M NaCl (final concentration) for 10 min at 30°C. Incubations were terminated by the addition of 2 volumes of ice-cold methanol-11.5 M HCl (100:1, v/v), this mixture was incubated on ice for 10 min, and lipids were extracted as described (18, 19).

Silanization

All glassware was silanized to minimize sample losses. Silanized autosampler vials and vial inserts were purchased from Alltech Associates. Other items were oven-dried (120°C, 60 min), silanized by standing in 5% dichlorodimethylsilane in toluene (60 min, room temperature) before transfer into 100% methanol for 30 min to deactivate unreacted reagent, and then air-dried.

Phosphoinositide extraction from platelets

To minimize the potential for acid hydrolysis, extractions were performed on ice. Three milliliters of ice-cold chloroform-methanol (1:2) containing appropriate internal standards [200 ng each of 16:0/16:0-PtdIns, PtdIns5 P , PtdIns4 P , PtdIns(4,5) P_2 , PtdIns(3,5) P_2 , and PtdIns(3,4,5) P_3] was added to platelets (0.8 ml) in polypropylene tubes (Sarstedt). After vigorous vortexing to ensure cell disruption, each sample was incubated on ice for 15 min. To induce a phase split, ice-cold chloroform (1 ml) was added, followed by 1 ml of 1.76% KCl, 100 mM citric acid, 100 mM Na $_2$ HPO $_4$, pH 3.6, 5 mM EDTA, and 5 mM tetrabutyl ammonium hydrogen sulfate. After mixing, the sample was incubated on ice for 5 min before centrifugation (200 g, 5 min) to complete the phase split. The lower, organic phase was transferred into a clean silanized tube using a silanized pipette. The upper, aqueous phase was reextracted with 2 ml of synthetic lower phase, and the resultant lower phase was combined with the previous lower phase extract. Two milliliters of 0.88% KCl was added to the remaining aqueous phase before extraction with 2 ml of water-saturated butanol (30 min incubation on ice). The resultant butanol-rich upper phase was combined with the previous lower phase extracts. After drying, the sample was resuspended in 50 μ l of chloroform-methanol-water (5:5:1) and transferred into a silanized autosampler vial insert ready for LC-MS analysis.

LC-MS analysis

Gradient separation of phosphoinositides (1 μ l injection) on Luna silica2 (3 μ m, 1.0 \times 150 mm; Phenomenex) used 100% chloroform-methanol-water (90:9.5:0.5, v/v) containing 7.5 mM ethylamine (solvent A) held for 2 min before changing to 45% acetonitrile-chloroform-methanol-water (30:30:32:8, v/v) containing 10 mM ethylamine (solvent B) over 1 min, then to 55% solvent B over 17 min at 100 μ l/min, before recycling back to solvent A over 1 min at 140 μ l/min and held for 14 min to reequilibrate. Detection was by negative electrospray ionization on a Shimadzu QP8000 α single quadrupole mass spectrometer (probe voltage, -4 kV; nebulizer gas, 4 l/min N $_2$; desolvation line temperature, 300°C; or, when optimized for [M-2H] $^{2-}$ ions, -3 kV, 2.5 l/min, and 155°C) or a Bruker Daltonics HCTplus Ion Trap mass spectrometer (instrument settings as for infusion analysis). Quantification was based on peak area of the molecular ion relative to the peak area for the corresponding 16:0/16:0 internal standard.

MS n infusion analysis

Samples in solvent A were infused at 2, 4, or 10 μ l/min using a Cole Palmer 74900 series infusion pump into a Bruker Daltonics HCTplus Ion Trap mass spectrometer (capillary voltage, -4 kV; nebulizer gas, 4 l/min N $_2$; nebulizer pressure, 10 p.s.i.; source temperature, 300°C). The pump and lines were washed with 1 ml of solvent B between samples.

RESULTS

Phosphoinositide detection and identification at high sensitivity in small biological samples, often derived from

$<5 \times 10^6$ cells, requires that all parts of the analysis must be optimized. To this end, we have investigated each stage from extraction to final analysis as outlined below.

Silanization

Use of silanized glassware is essential for the extraction, transfer, and storage of polyphosphoinositides because these highly polar lipids stick avidly to untreated surfaces. This problem is compounded when these lipids are present at trace quantities, as in real biological samples, because if the number of active sites capable of binding the lipid exceeds the number of lipid molecules capable of acting as binding partners, then little or no lipid will be detected, even if it was present in the original sample. Glassware silanization increased recovery by $>50\%$ for various highly polar lipids, including lysophosphatidate, sphingosine 1-phosphate, PtdIns P_2 , and PtdIns P_3 . Silanization only increased PtdIns recovery by $\sim 5\%$. It should be noted that silanization itself is slowly reversible, particularly in the presence of acid. Surprisingly, extraction in plastic tubes (e.g., 15 ml polypropylene centrifuge tubes from Sarstedt) gave significantly better recoveries for these very polar lipids than untreated glass and only slightly poorer than with silanized glass. Because stable silanization of components within the LC-MS apparatus that come into sample contact is not possible, ideally all surfaces should be an inert material such as PEEK. This was readily achieved for the liquid chromatograph plumbing by replacing all appropriate tubing with narrow-bore PEEK, kept as short as possible to minimize interactions. Because inositol polyphosphates are chelating agents that avidly bind metals (20), a similar interaction between the phosphoinositides and stainless steel components may also occur. Therefore, replacement is desirable wherever possible (but note that column walls are normally stainless steel).

Phosphoinositide extraction

Ordinary Bligh and Dyer- or Folch-based lipid extraction protocols give poor recoveries of polyphosphoinositides and other highly polar acidic lipids. Acidified extraction improves phosphoinositide recovery by protonating the phosphate groups, thereby disrupting their ionic interactions with cellular proteins and increasing solubility in organic solvents. Most published procedures use acidification with HCl; however, we found substantial and quite variable losses of higher phosphoinositides. In efforts to overcome these problems, we made various modifications, including extractions with lower HCl concentrations, ammonia neutralizations, and replacing HCl with formic acid; however, only small improvements were achieved and recoveries remained variable. This was probably attributable in large part to how much water came across with the organic phase when it was collected after the phase split: most acid partitions into the upper aqueous phase, although the small amount in the organic phase (together with any upper phase accidentally carried over) is concentrated upon drying, readily causing phosphoinositide deacylation and possibly phosphate loss. Samples

dried very rapidly are less susceptible to hydrolysis than those dried more slowly. Although rapid drying improved recovery, sample stability remained poor even after resuspension in injection solvent, probably as a result of trace amounts of HCl remaining. Indications of sample hydrolysis were higher levels of lysophosphatidates eluting slightly ahead of PtdIns P and the presence of lysophosphoinositides derived from the 16:0/16:0 internal standards. Sample warming increased the losses. Because removal of acid was a problem, we acidified with citric acid/sodium phosphate buffer, pH 3.6, as was used for lysophosphatidate extraction (21). This largely removed the problems of acid hydrolysis; however, the PtdIns(3,4,5) P_3 in particular did not readily partition into the lower organic phase. A back-extraction of the aqueous phase with synthetic lower phase improved recovery, whereas a second reextraction of the aqueous phase, this time with water-saturated butan-1-ol, recovered much of the remaining phosphoinositide. The drawback with butanol extraction is the substantial carry-over of salts, which interfere particularly with the analysis of PtdIns(3,4,5) P_3 and cannot be readily removed without further loss of this lipid. For this reason, we performed only a single, low-volume butanol extraction on each sample.

The incorporation of the ion pair agent tetrabutylammonium hydrogen sulfate increased recoveries by improving the solubility of the polyphosphoinositides, particularly PtdIns(3,4,5) P_3 , in organic solvents, as noted previously (22). The KCl increases the ionic strength of the aqueous phase and thus also helps drive lipids into the organic phase. Using this procedure, recoveries of phosphoinositide standards, including PtdIns(3,4,5) P_3 , from spiked biological samples exceeded 90% compared with direct injection of the same, nonextracted standards onto the LC-MS apparatus. These high efficiencies indicate that any possible metal chelation by the polyphosphoinositides did not adversely affect their recoveries.

LC separation

To resolve the intact phosphoinositides, a liquid chromatograph coupled directly to a MS detector was adopted. Initial efforts to develop a method for the complete LC resolution of phosphoinositides were adapted from the PtdIns $4P$ and PtdIns(4,5) P_2 separation method of Gunnarsson et al. (23) published before the recognition of other regioisomers as biologically important. We had used a similar LC gradient for separation of the main membrane phospholipids (phosphatidate, PtdIns, phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylserine, and sphingomyelin) on silica (24) and noted that it could also separate PtdIns $4P$ and PtdIns(4,5) P_2 . However, with these chloroform-methanol-water-ammonia solvent mixtures, polyphosphoinositide peak shape was often poor and sensitivity was very low (detection limit for PtdIns P_2 is >100 pmol on column). Numerous solvent combinations were tested to improve these parameters, but the chloroform-methanol-water mixes remained the best. Including acetonitrile in solvent B slightly improved the MS signal intensity for some lipids, probably by reducing the amount of methanol

required, because higher methanol concentrations can cause some signal suppression.

The choice of mobile phase modifiers, although essential to achieve good chromatographic peak shape, is highly constrained by the need for volatility and the sensitivity of electrospray ionization to ion suppression, which generally limits concentration to <20 mM. As noted previously (23), acidic elution conditions result in substantial phosphate loss, whereas this was not apparent under the alkaline conditions used here because no conversion of phosphoinositide standards to less phosphorylated structures was detected. Neither was on-column phosphate isomerization or deacylation of phosphoinositide standards (forming the equivalent lyso lipid) detected. Replacing ammonia with several different alkylamines showed that ethylamine, with its higher proton affinity and high volatility, gave better peak shape than ammonia and was effective at lower concentrations, routinely 5–10 mM compared with >50 mM. Its main drawback is its high alkalinity (~pH 12 at 10 mM in water), as this results in a gradual dissolution of the silica. We found that this occurred slowly provided that the water content of the LC solvent mix was kept low (routinely, columns lasted for >1,000 injections before resolution changes or high back-pressures necessitated their replacement). Extended exposure to >5% water caused the rapid generation of silica fines as a result of column degradation, leading to tubing blockages. Brief exposure (at the top of gradients) of up to 8% water did not greatly increase the rate of column degradation, although solvent mixes with 10% or more water resulted in many blockages.

Attempts to find alternative column chemistries with greater stability than silica, yet similar capacity to resolve the lipid classes, proved unsuccessful, with none giving as complete separation or as good peak shape. The quality of the silica is critical for optimal resolution, particularly of the more polar acidic lipids such as the phosphoinositides. The best silica is uniformly spherical with narrow particle size distribution, high surface area, and very low metal content; nevertheless, apparently identical columns from the same manufacturer will show different resolutions.

The final solvent mixes routinely adopted for the separation of phospholipids, including the phosphoinositides, were chloroform-methanol-water (90:9.5:0.5) containing 7.5 mM ethylamine (solvent A) and acetonitrile-chloroform-methanol-water (30:30:32:8) containing 10 mM ethylamine (solvent B). A typical separation of 16:0/16:0-phosphoinositide standards [PtdIns3P+PtdIns4P, PtdIns5P, PtdIns(4,5)P₂, PtdIns(3,4)P₂, PtdIns(3,5)P₂, and PtdIns(3,4,5)P₃] is shown in **Fig. 1**. We expected PtdIns(3,4,5)P₃ to elute after the PtdInsP₂ species; however, surprisingly, PtdIns(3,4,5)P₃ normally eluted with the corresponding PtdIns(3,5)P₂ species. Under optimal conditions, PtdIns5P eluted slightly ahead of the corresponding PtdIns3P and PtdIns4P molecular species (**Fig. 1**, inset). It was not possible to sufficiently resolve intact PtdIns3P and PtdIns4P chromatographically for routine analytical purposes, although PtdIns3P did lead the equivalent PtdIns4P species. This was most readily detected by overlaying the [M-H]⁻ and [M-2H]²⁻ profiles, because these two regioisomers show

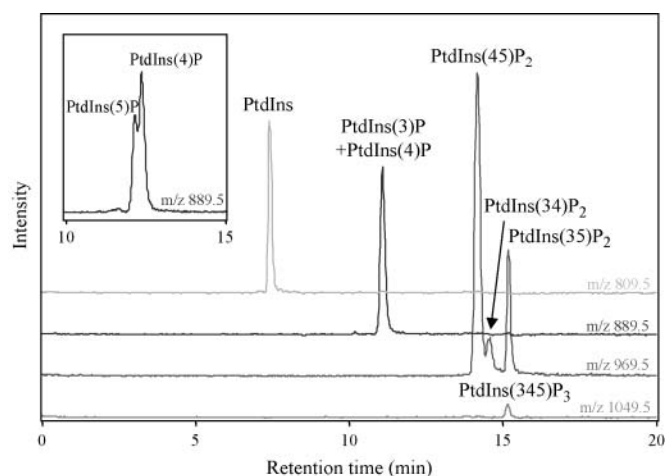


Fig. 1. Liquid chromatography-mass spectrometry (LC-MS) separation of 16:0/16:0-phosphoinositides. Detection as singly deprotonated [M-H]⁻ ions at *m/z* 809.5, 889.5, 969.5, and 1,049.5. Although identical molecular species of phosphatidylinositol 3,4,5-trisphosphate (PtdIns3P) and PtdIns4P coelute, PtdIns5P will partially resolve (inset). Different fatty acid combinations cause a small shift in retention time, with longer acyl chain, more unsaturated structures eluting ahead of shorter chain, more saturated structures. The doubly deprotonated [M-2H]²⁻ and sodiated [M-2H+Na]⁻ ions were also observed for PtdInsP, PtdInsP₂, and PtdInsP₃, although their intensities relative to the corresponding [M-H]⁻ ions were low under the MS conditions used here (Shimadzu QP8000α, 300°C, -4 kV, 4 l/min). Other ions, including metal adducts, were only seen at trace levels.

slightly different ratios between the singly and doubly deprotonated ions, with the latter being most readily formed from PtdIns4P. Differences in acyl structure cause small differences in retention time, with shorter chain, more saturated acyl structures eluting later than longer chain, more unsaturated structures. Hence, 18:0/20:4-phosphoinositides elute ahead of the equivalent 16:0/16:0 structures.

Subtle differences in solvent composition affected resolution; thus, each new batch of solvent needed characterizing with authentic lipid standards before attempting to resolve phosphoinositides from biological samples. The silica column usually took at least three run cycles before reasonable retention time stability was achieved. Sample carryover between runs was sometimes observed, particularly if there was a large amount of polyphosphoinositide in the preceding run; however, running the solvent gradient to a higher polarity than required to elute PtdInsP₃ coupled with rigorous autosampler needle washing (particularly the external surface) between injections greatly reduced this contamination. If necessary, a blank run between samples removed carryover.

Mass spectrometry

Using a Shimadzu QP8000α single quadrupole mass spectrometer, most glycerophospholipids give their strongest ion intensities when using a desolvation line temperature of 300°C. This was also true for the singly deprotonated [M-H]⁻ polyphosphoinositide ions, although ion intensity became progressively weaker as phosphorylation increased,

becoming a particular problem for PtdIns(3,4,5)P₃ detection. Optimization of phosphoinositide ion intensities demonstrated that the doubly deprotonated [M-2H]²⁻ ion, although normally minor at 300°C, became the predominant ion at lower temperatures, with a maximum at ~155°C, at which its intensity was 5–10 times greater than that for the corresponding [M-H]⁻ ion at 300°C. The [M-H]⁻ intensity was reduced by only ~50% at 155°C compared with 300°C. Presumably, the lower temperature stabilized the higher ionization states. Thus, when detecting PtdInsP₂ and PtdInsP₃, the lower desolvation line temperature significantly improved sensitivity, although at the expense of somewhat increased noise. Alterations in other parameters made relatively little difference, although a somewhat reduced probe voltage (-3 kV) and

lower nebulizer gas flow (2.5 l/min) slightly increased [M-2H]²⁻ intensities.

Ion trap mass spectrometry

The inability to completely chromatographically resolve the PtdInsP regioisomers led us to investigate whether the different isomers might show differential fragmentation patterns that could be used diagnostically. To this end, we ran a series of infusion analyses of authentic 16:0/16:0-PtdInsP standards on a Bruker Daltonics HCTplus Ion Trap mass spectrometer. Early indications suggested that rapid phosphate loss from the inositol ring would prevent differential fragmentations, and this certainly was the case when fragmenting the singly deprotonated [M-H]⁻ mo-

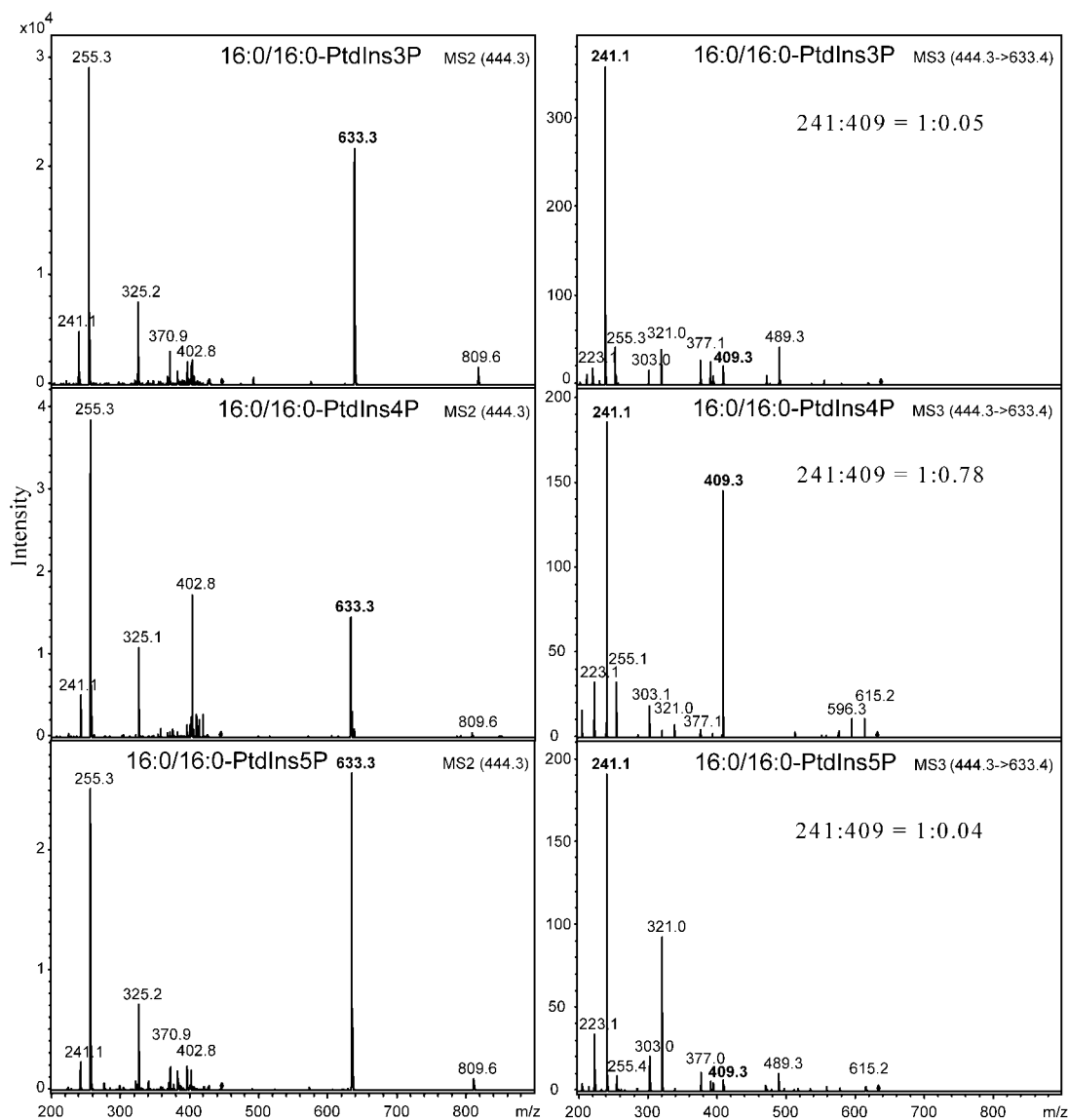


Fig. 2. Diagnostic MS² and MS³ fragment spectra of 16:0/16:0-PtdIns3P, -PtdIns4P, and -PtdIns5P. Samples at 2.5 µg/ml in solvent A were infused at 4 µl/min into a Bruker Daltonics HCTplus Ion Trap mass spectrometer. MS² fragmentation of the doubly deprotonated [M-2H]²⁻ ion at m/z 444 generates the [M-H-RCOOH]⁻ ion at m/z 633, which, when fragmented, gives the diagnostic MS³ pattern containing the [inositol monophosphate-H₂O-H]⁻ ion at m/z 241 and the m/z 409 ion resulting from the loss of 224 Da. The ratio of m/z 241 to m/z 409 ion intensities is diagnostic for the relative proportions of PtdIns4P and PtdIns3P + PtdIns5P in a sample.

lecular ion. However, the doubly deprotonated $[M-2H]^{2-}$ ion showed a very different result. PtdIns3P and PtdIns5P gave essentially identical fragmentations at MS^2 , MS^3 , and MS^4 , however, PtdIns4P gave a very different pattern at MS^3 (Fig. 2). Although some small differences were evident at MS^2 , these were insufficiently consistent for diagnostic purposes with biological samples.

Fragmentation of the $[M-2H]^{2-}$ ion (MS^2) formed a major ion, $[M-H-RCOOH]^-$, through the loss of one acyl chain (m/z 633 in the case of 16:0/16:0-PtdInsP). This ion, when fragmented further (MS^3), generated a primary [inositol phosphate- H_2O-H] $^-$ ion at m/z 241 and a characteristic amount of another ion retaining a fatty acid (m/z 409 for 16:0 after the loss of 224 Da from the parent m/z 633 ion). Further fragmentation of m/z 409 (MS^4) produced a single ion at m/z 152.6, although signal intensity was extremely low.

16:0/16:0-PtdIns4P produced high levels of m/z 409, whereas 16:0/16:0-PtdIns3P and PtdIns5P generated very little m/z 409. The resulting 241:409 ratio was diagnostic for the 16:0/16:0-PtdInsP regioisomer: samples with diagnostic ratios approaching 1:1 contained PtdIns4P with essentially no PtdIns3P/PtdIns5P, whereas low ratios of $\sim 1:0.05$ indicated the opposite.

This diagnostic ratio analysis was also applicable to 18:0/20:4-PtdInsP, in which two dissimilar fatty acids make the fragmentation slightly more complex (Fig. 3). Two fragments resulting from the loss of 18:0 (m/z 681) or 20:4 (m/z 661) were seen at MS^2 . By separately trapping these ions and inducing their further fragmentation (MS^3), resultant ions were detected, after the loss of 224 Da, at m/z 457 and 437, respectively. As with 16:0/16:0-PtdInsP, further fragmentation of these ions (MS^4) detected a single ion at m/z 152.6. By averaging the 241:437 (for 18:0) and 241:457 (for 20:4) ratios, a diagnostic ratio essentially identical to the 241:409 ratio for the equivalent 16:0/16:0 species was obtained. The diagnostic ratios were reproducible over a range of infusion rates and concentrations down to <500 pg infused/min, although at lower values the ratios started to converge. By plotting the calibration curve of the diagnostic ratio against PtdIns4P versus PtdIns3P + PtdIns5P composition (Fig. 4), it was possible to determine relative PtdInsP composition in an unknown sample from the calculated diagnostic ratio for a particular PtdInsP molecular species in that sample. Coupling this approach with the ability to chromatographically distinguish PtdIns3P and PtdIns5P enabled us to determine levels of each PtdInsP.

Biological sample analysis

Coupling a fast ion trap mass spectrometer with LC (LC-MS n), which partially resolves PtdIns5P from the other two regioisomers, enabled this technique to determine the relative amounts of the different PtdInsP, PtdInsP $_2$, and PtdInsP $_3$ in yeast and human platelet samples. The major diacyl PtdInsP and PtdInsP $_2$ species in the yeast *Saccharomyces cerevisiae* are 16:0/16:1, 16:0/18:1 + 18:0/16:1, and 18:0/18:1 (Fig. 5A). Salt-induced hyperosmotic stress did

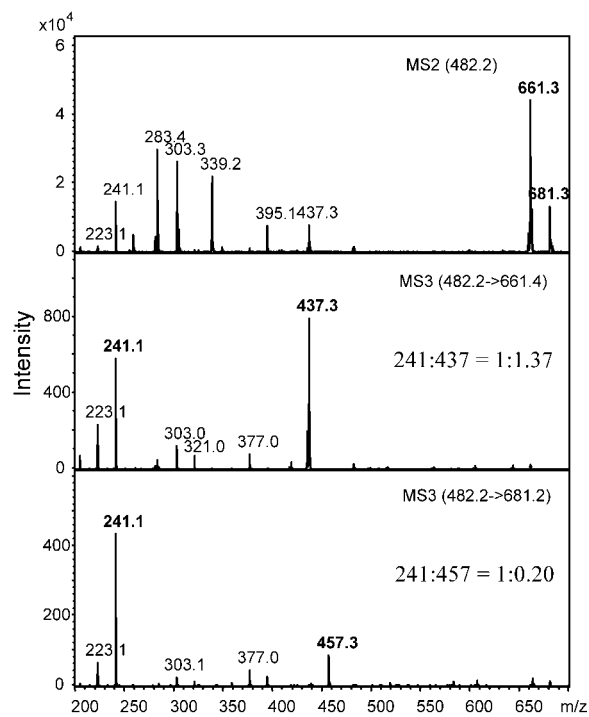


Fig. 3. Diagnostic MS^2 and MS^3 fragmentation of 18:0/20:4-PtdIns4P. Sample (2.5 $\mu\text{g}/\text{ml}$) was infused at 4 $\mu\text{l}/\text{min}$. MS^2 fragmentation of the doubly deprotonated $[M-2H]^{2-}$ ion at m/z 482 generated two $[M-H-RCOOH]^-$ ions at m/z 661 and 681 resulting from the loss of the 20:4 and 18:0 fatty acids, respectively. Individual MS^3 fragmentation of these ions generated the m/z 241 ion together with one at m/z 437 or 457 after the loss of 224 Da. Averaging the two ratios $[(241:437 + 241:457)/2]$ gives a diagnostic ratio (1:0.79) that is essentially identical to that for pure 16:0/16:0-PtdIns4P.

not change the relative proportions of these species, although it clearly induced PtdIns(3,5)P $_2$ formation in wild-type yeast cells (Fig. 5B). No PtdIns(3,4,5)P $_3$ or PtdIns5P formation (as indicated by peak splitting) was detected. Only traces of PtdIns(3,4)P $_2$ were seen. A diagnostic ratio

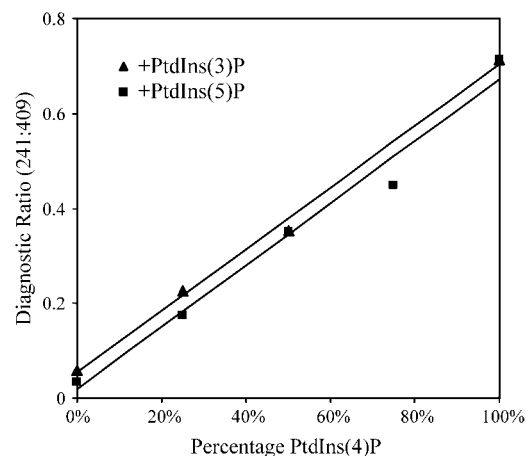


Fig. 4. Diagnostic ratios for different 16:0/16:0-PtdIns4P/PtdIns3P and -PtdIns4P/PtdIns5P mixtures. Calibration samples (2.5 μg lipid/ ml) were infused at 4 $\mu\text{l}/\text{min}$ (10 ng/min) into a HCTplus Ion Trap mass spectrometer.

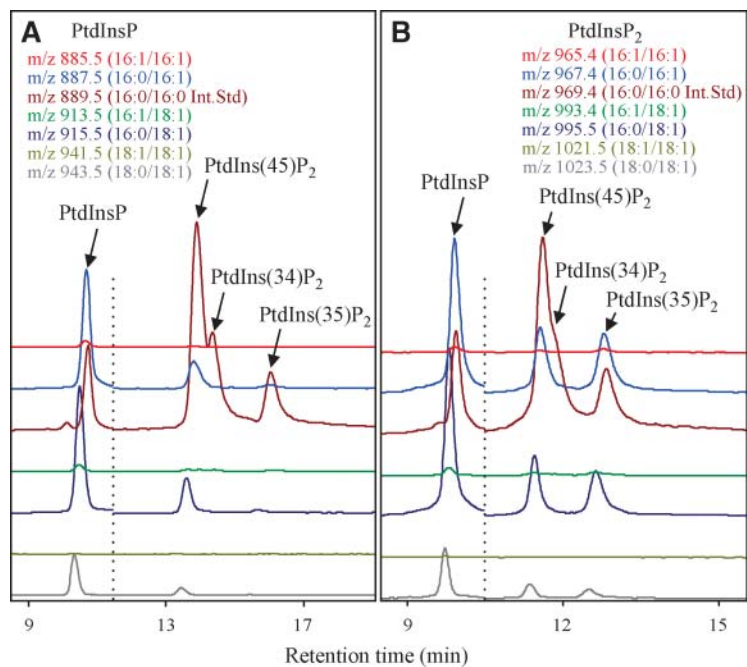


Fig. 5. *Saccharomyces cerevisiae* PtdInsP and PtdInsP₂. A: Wild-type control cells. B: Hyperosmotically (salt)-stressed cells. Both panels show the main phosphoinositide species in yeast (as [M-H]⁻ ions) together with 16:0/16:0-PtdIns3P and -PtdIns(4,5)P₂, -PtdIns(3,4)P₂, and -PtdIns(3,5)P₂ internal standards, as detected with a Shimadzu QP8000α mass spectrometer.

of 0.38 was calculated for 16:0/16:1-PtdInsP species in control cells, indicating a composition of 51% PtdIns4P and 49% PtdIns3P. The other acyl species gave similar values. After salt stress, a ratio of 0.33 was obtained, indicating that the proportion of PtdIns3P had increased to 58%. Total PtdIns4P mass did not change significantly upon hyperosmotic stress (from 217 ± 34 to 205 ± 34 pmol/ 10^9 cells), but there was an increase in PtdIns3P (from 209 ± 32 to 288 ± 46 pmol/ 10^9 cells). PtdIns(4,5)P₂ mass almost doubled (from 59 ± 28 to 106 ± 14 pmol/ 10^9 cells), whereas PtdIns(3,5)P₂ quintupled (from 22 ± 14 to 119 ± 16 pmol/ 10^9 cells).

The major PtdInsP and PtdInsP₂ acyl structure in human platelets is 18:0/20:4, representing >70 mol% of the

total molecular species (**Fig. 6A**). This profile is essentially identical to that for PtdIns and does not appear to change after thrombin stimulation. No PtdIns5P was detected. The PtdInsP peak consisted of 79% PtdIns4P and 21% PtdIns3P in resting platelets, but the latter became undetectable after 2 min of thrombin stimulation, although total PtdInsP mass did not change (13.8 ± 3.4 and 13.5 ± 3.5 nmol/ 10^9 platelets, respectively). Unusually for a mammalian cell, PtdInsP levels were ~2.5 times higher than those for PtdIns (5.2 ± 1.6 nmol/ 10^9 platelets). In resting platelets, PtdIns(4,5)P₂ and small amounts of PtdIns(3,4)P₂ and PtdIns(3,5)P₂ were detected ($1,240 \pm 127$, 80 ± 6 , and 36 ± 25 pmol/ 10^9 platelets, respectively). After 2 min of thrombin stimulation, all PtdInsP₂ isomers increased

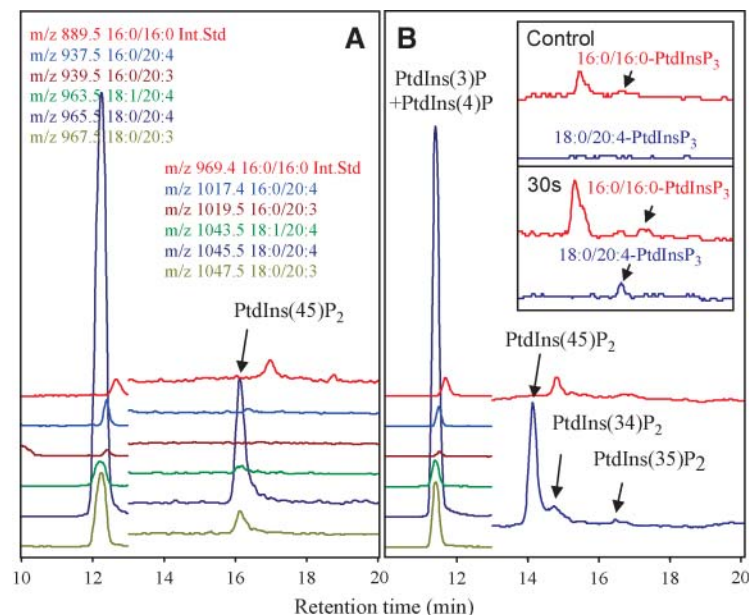


Fig. 6. Human platelet phosphoinositides. A: Unstimulated platelets. B: Platelets stimulated with thrombin for 2 min (1 U/ml). Both panels show the main PtdInsP and PtdInsP₂ molecular species in platelets plus 16:0/16:0-phosphoinositide internal standards. The inset shows 18:0/20:4-PtdIns(3,4,5)P₃ formation (together with 16:0/16:0 internal standard) in unstimulated (below detection limits) and 30 s thrombin-stimulated platelets detected as [M-2H]²⁻ ions with a desolvation line temperature of 155°C. All other phosphoinositides were detected as [M-H]⁻ at 300°C.

(Fig. 6B) ($1,837 \pm 329$, 197 ± 91 , and 52 ± 33 pmol/ 10^9 platelets, respectively). 18:0/20:4PtdIns(3,4,5) P_3 was undetectable in the control and 2 min thrombin-stimulated samples; however, it was detectable at 15 s and particularly after 30 s of thrombin stimulation (Fig. 6B, inset), when it increased to 77 ± 35 pmol/ 10^9 platelets.

The sensitivity of this procedure has enabled minimum LC-MS detection limits (signal/noise > 3) of ~ 100 fmol of PtdIns, 250 fmol of PtdIns P , 1 pmol of PtdIns P_2 , and 5 pmol of PtdIns P_3 on column using the QP8000 α mass spectrometer in selected ion monitoring mode. This has allowed us to obtain data for total PtdIns P and PtdIns(4,5) P_2 with samples derived from as few as 10,000 cells, although routinely we would aim to use material from $>10^6$ cells, particularly if isomeric analysis of PtdIns P was required.

DISCUSSION

Efforts to develop a highly sensitive means of resolving and quantifying intact phosphoinositides from real biological samples, without prior derivatization or other modification such as deacylation, have proved frustratingly elusive. Various factors all contribute to this difficulty: low to extremely low abundance; poor extraction from the biological matrices, particularly of the more highly phosphorylated forms; adsorption to extraction vessel walls and other surfaces; inability to resolve the different regioisomers; lack of sufficiently sensitive detection methods. We have developed LC-MSⁿ and associated techniques that largely overcome these problems and provide, for the first time, an approach that can relatively rapidly characterize and quantify the native phosphoinositide composition, including the individual regioisomers and their component molecular species, in real biological samples. Although the use of a liquid chromatograph coupled to a single quadrupole mass spectrometer is sufficient for many analyses, including isomeric identification and quantification of PtdIns P_2 , a fast ion trap mass spectrometer is essential if isomeric identification of PtdIns P is required. These techniques also allow us to investigate the possibility of other phosphoinositide isomers with phosphate groups on the 2 and 6 positions of the inositol ring. Although no one has identified such structures, we are unaware of reasons why they could not be formed, although steric hindrance by the diacylglycerol part of the structure may make phosphorylation at these sites more difficult. Certainly, free inositol phosphates are found with phosphorylation at these positions.

The poor and highly variable recoveries of phosphoinositides when using HCl-acidified extraction have not been noted in previously published procedures, probably because most continue with methylamine deacylation before identification and quantification of the phosphoinositol head groups by ion-exchange chromatography. Partial deacylation during the extraction makes no difference to this type of analysis and thus would not have been detected. Milder acidification with a citric acid/

sodium phosphate buffer, pH 3.6, which does not so readily dissolve in organic solvent, coupled with an initial chloroform-based extraction followed by butan-1-ol reextraction, greatly improved recovery, reproducibility, and sample stability, although losses may still occur with longer storage.

The PtdIns P fragmentation patterns share similarities with those predicted by Hsu and Turk (16); however, the loss of 224 Da from the $[M-H-RCOOH]^-$ ion, which we saw at MS^3 , was not described. In agreement with our own observations, they also found that PtdIns4 P more readily formed $[M-2H]^{2-}$ ions than PtdIns3 P . PtdIns3 P and PtdIns5 P show a similar propensity to form $[M-2H]^{2-}$ ions. Attempts to improve signal intensity using postcolumn addition of piperidine, as used by other groups (13, 25), proved a failure and, in our system, actually reduced signal intensity. The ethylamine, which is essential for good ionization, itself improved on the piperidine, especially as it is effective at a lower concentration and thus reduces ion suppression effects.

The use of 16:0/16:0-phosphoinositides as internal standards is not ideal for organisms such as yeast, which have a very limited fatty acid composition and in which small amounts of 16:0/16:0 species are formed naturally; however, only a very limited range of structures are currently available commercially. In mammalian cells, this is not normally an issue, because almost all polyphosphoinositides are polyunsaturated, predominantly 18:0/20:4, with only trace levels of 16:0/16:0 species. Although it has been suggested that the PtdIns(3,4,5) P_3 molecular species profile may change depending on the stimulus (13), we have found no evidence for this, at least in thrombin-stimulated platelets, in which 18:0/20:4 is the major structure, as it is with the other phosphoinositides.

In conclusion, the methodology described here allows, for the first time, a rapid, sensitive, and comprehensive analysis of all currently known phosphoinositides without prior fractionation or modification and as such enables their routine profiling, which will be of great benefit in the study of these molecules in their many roles. ■

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