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Separation and detection of all phosphoinositide isomers by ESI-MS

Sabine Kiefer[a], Johannes Rogger[a], Anna Melone[b], Ann C. Mertz[b], Anna Koryakina[a], Matthias Hamburger[a], Peter Küenzi[a,*]

[a] Institute of Pharmaceutical Biology, University of Basel, Klingelbergstrasse 50, 4056 Basel, Switzerland
[b] Institute of Biochemistry and Genetics, Department of Biomedicine, University of Basel, Mattenstrasse 28, 4058 Basel, Switzerland

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ABSTRACT

Phosphoinositides (PIs) play fundamental roles as signalling molecules in numerous cellular processes. Direct analysis of PIs is typically accomplished by metabolic labelling with ^{3}H-inositol or inorganic ^{32}P followed by deacylation, ion-exchange chromatography and flow scintillation detection. This analysis is laborious, time-consuming, and involves massive amounts of radioactivity. To overcome these limitations we established a robust, non-radioactive LC–ESI–MS assay for the separation and analysis of deacylated PIs that allows discrimination of all isomers without the need for radioactive labelling. We applied the method to various cell types to study the PI levels upon specific stimulation.

1. Introduction

The inositol containing glycerophospholipids, collectively known as phosphoinositides (PIs), play a fundamental role in diverse cellular functions such as cell growth and differentiation, motility, calcium mobilisation and oncogenesis [1,2]. The family of the phosphoinositides consists of the non-phosphorylated precursor phosphatidylinositol (PtdIns) and seven derivatives with different phosphorylation patterns on the myo-inositol ring, where the 3-, 4- and 5-positions can be phosphorylated by specific kinases (Fig. 1A).

PIs derived from PtdIns and its phosphorylation products phosphatidylinositol-4-phosphate (PtdIns4*P*) and phospha tidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) form the so called canonical pathway [3] and are believed to be kept at constant levels at the plasma membrane. The other PIs are considered to be low-abundant signalling molecules that transiently appear upon stimulation. Stimulation with growth factors or insulin leads to increased PtdIns(3,4,5)P_3 levels, which in turn produces specific cellular responses. The bisphosphorylated PIs containing PtdIns(3,4)P_2, PtdIns(4,5)P_2, and PtdIns(3,5)P_2 moieties play distinct roles in signal prolongation after PtdIns(3,4,5)P_3 inducing stimuli, regulation of the actin cytoskeleton and vesicle transport, respectively [4–8]. PIs containing phosphoinositide monophosphates were long thought to be mere intermediates in the pathway but are now recognised to possess specific functions by themselves in protein sorting, vesicular trafficking and in osmotic stress response [9–12].

Analysis of PIs has been achieved in several ways. Most frequently, metabolic radioisotope labelling with inorganic ^{32}P or ^{3}H-inositol, lipid extraction, hydrolysis followed by chromatographic separation and radiographic analysis of phosphoinositides has been used [13–15]. Metabolic labelling involves very high doses of radioactivity (GBq), long labelling times and only detects the turnover of PIs, whereas dormant pools of PIs remain unlabelled. More recent approaches include fluorescent-labelled binding proteins for specific PIs, and antibodies directed against PIs [16,17]. However, differentiation of all the mono- and bisphosphorylated positional isomer PIs, has not been achieved yet.

Two fundamentally different approaches have been pursued in PI analysis: (i) a comprehensive profiling of intact PIs [18–21] and (ii) head group analysis after cleavage of the lipid moieties [22,23]. The first approach used in lipidomics leads to a highly complex picture due to a plethora of closely related molecules that only vary in their lipid moieties. This approach including quantification has been successfully applied to cells producing phophoinositides with limited complexity in their lipid residues, such as platelets [24–26]. Even though the analysis of intact PIs would be the preferred approach, it is currently unsuitable for most cells due to the overwhelming complexity of their PI patterns. Therefore, separation and detection of the head groups following deacylation (Fig. 1B) is a more suitable approach if the focus lies on the detec-

* Corresponding author. Present address: CSL Behring, Wankdorfstrasse 10, 3022 Bern, Switzerland. Tel.: +41 31 344 40 48.
E-mail addresses: peter.kueenzi@cslbehring.com, peter.kueenzi@unibas.ch (P. Küenzi).

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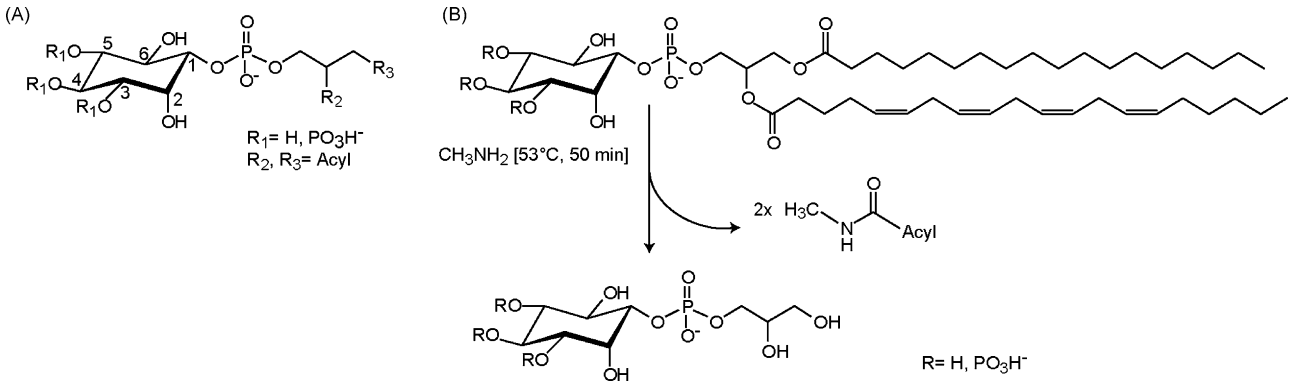

Fig. 1. (A) Structure of a typical PtdIns, sn-1-stearoyl-2-arachidonyl-phosphatidylinositol and the performed deacylation step, resulting in cleavage of the lipid moiety. (B) Structure of phosphatidylinositol-3,4,5-trisphosphate (PtdInsP_3); numbering of the myo-inositol ring is indicated.

tion of the change in phosphorylation pattern that exerts the major influence on the subsequently elicited signalling. Given the low abundance of certain PIs, even the analysis of deacylated lipids is highly challenging.

As alternatives to radioisotope labelling, analysis by mass spectrometry [27], suppressed conductivity detection [28] and evaporative light scattering detection (ELSD) [29] have been used. Unfortunately, none of these allowed a discrimination of all isomers. An LC–ESI–MS method for separation of deacylated PtdInsP_2 isomers has been recently published [14]. However, chromatography suffered from poor peak shape and co-elution of PtdIns(3,5)P_2 and PtdIns(3,4,5)P_3.

Since the PtdIns moieties of PIs can be interconverted by specific kinases and phosphatases, inhibition, stimulation, modification, or deletion of one of these enzymes may have profound implications on the biological response. Therefore, separation and simultaneous detection of all headgroups of PIs is of major importance for a better understanding of their biological roles, and a robust and sensitive method is of general interest to researchers involved in cell signalling.

2. Experimental

2.1. LC–MS instrumentation

HPLC separation was carried out on a series 1100 system equipped with degasser, binary high pressure mixing pump, and column thermostat (Agilent Technologies). A liquid handler 215 (Gilson) was used as autosampler. The HPLC was coupled to an Esquire 3000 ion trap mass spectrometer equipped with an electrospray (ESI) interface (Bruker Daltonics). Data acquisition and processing was performed using HyStar 3.0 software from Bruker Daltonics.

2.2. LC–MS method

2.2.1. Ion-pair chromatography

N,N-dimethylhexylamine (DMHA; Acros, Thermo Fisher) was used as ion-pair reagent. Mobile phase A consisted of water containing 5 mM DMHA and 4 mM glacial acetic acid (Sigma–Aldrich) and mobile phase B of acetonitrile or methanol with 5 mM DMHA and 4 mM glacial acetic acid. All solvents were from Scharlau (Scharlau, Barcelona, Spain).

2.2.2. Columns

Various columns were tested for suitability in phosphoinositide analysis, including Atlantis C18 (150 mm × 4.6 mm, 5 μm) and T3 (150 mm × 3.5 mm, 3 μm; Waters, Baden, Switzerland), Nucleosil C100 (250 mm × 4.6 mm, 5 μm; Macherey-Nagel, Düren, Germany), liChrospher diol (125mm × 4.0 mm, 5 μm; Merck, Darmstadt, Germany) and Aqua C18 (250 mm × 4.6 mm, 5 μm and 75 mm × 2.0 mm, 3 μm; Phenomenex, Torrance, CA).

2.2.3. Separation of phosphoinositides

2.2.3.1. Method 1. Separation of deacylated PIs with different numbers of phosphorylations; PtdIns, PtdIns*P*, PtdInsP_2 and PtdInsP_3, was achieved by ion-pair chromatography on a Aqua C18 column (3 μm, 125 Å, 75 mm × 2.0 mm). A gradient from mobile phase B (acetonitrile) 0.1 to 50% in 25 min and a wash step (50% B to 100% B in 3 min, 100% B for 12 min, 100% B to 0.1% B in 5 min, 0.1% B for 5 min) was applied.

2.2.3.2. Method 2. Separation of deacylated PIs with different numbers of phosphorylation plus additional separation of phosphoinositides bisphosphate isomers PtdIns(4,5)P_2, PtdIns(3,5)P_2, PtdIns(3,4)P_2, was achieved by ion-pair chromatography on an Aqua C18 column (125 Å, 250 mm × 4.6 mm, 5 μm). A gradient from mobile phase B (acetonitrile) 15 to 35% in 40 min followed by a wash sequence (35% B to 100% B in 2 min, 100% B for 15 min, 100% B to 15% B in 3 min, 15% B for 5 min) was applied.

2.2.3.3. Method 3. Separation of PtdIns(3)*P*, PtdIns(4)*P* and PtdIns(5)*P*, additionally to separation of all other PIs, was performed with methanol as mobile phase B and a gradient from 15 to 50% in 60 min, followed by a wash step (50% B to 100% B in 2 min, 100% B for 15 min, 100% B to 15% B in 3 min, 15% B for 5 min).

2.3. Mass spectrometry

Negative ion LC–MS spectra on the ion trap instrument were recorded after optimization of settings, under ion charge control conditions (ICC 20000) at a scan speed of 13,000 *m*/*z*/s, using a gauss filter width of 0.2 *m*/*z*. Nitrogen was used as a drying gas at a flow rate of 10 l/min and as a nebulizing gas at a pressure of 30 psi. The nebulizer temperature was set to 300 °C. Spectra were recorded in the range of *m*/*z* 200–600 in negative mode. Capillary voltage was at 4500 V, endplate offset at −500 V, capillary end voltage at −115.0 V, skimmer voltage −40.0 V and trap drive at 53.4.

2.4. Flow scintillation analysis

Levels of radioactively labelled intracellular phosphatidylinositides were determined essentially as described [30]. Briefly, 4 million cells were incubated with 500 μCi $^{32}P_i$ for 60 min at 37 °C. After removal of non-incorporated $^{32}P_i$, cells were extracted as described below. The column effluent was splitted and exam-

ined online with a FLO-ONE A500 β-detector (Packard–Perkin Elmer).

2.5. Chemicals and cell culture

Murine bone marrow cells were cultured in Iscove's Modified Dulbecco's medium (IMDM; Sigma–Aldrich) supplemented with 10% heat-inactivated fetal calf serum (FCS; Amimed, Basel, Switzerland), 100 U/ml penicillin/streptomycin and 2 mM L-glutamine (both from Invitrogen, Basel, Switzerland). Cells were grown in humified atmosphere containing 5% CO_2 and maintained with 2 ng/ml recombinant murine interleukin-3 (IL3; PeproTech EC Ltd., London, UK).

Human embryonic kidney cells HEK 293 were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma–Aldrich) supplemented with 10% FCS, 100 U/ml penicillin/streptomycin and 2 mM L-glutamine. Cells were transfected with jetPEI cationic polymer transfection reagent (Polyplus-Transfection, Illkirch, France) according to the manufacturers' instructions. Twenty-four hour before transfection, cells were plated at 10^6 cells/25 cm^2 flask, then transfected with 2.6 μg GST-Vps34 and 0.4 μg Myc-S6K. Thirty hour after transfection, cells were starved over night and experiments were performed the following day.

Platelets were isolated from blood of healthy donors. Blood samples were mixed with acid citrate dextrose ACD (10.1 mM glucose, 30 μM citric acid, pH 6.5 in 0.9% NaCl, all from Sigma–Aldrich) and centrifuged for 5 min at 1000 × *g* [31]. Platelet rich plasma was collected and washed in PBS.

Chemicals used for experiments were: adenosine (Ade), N-formyl-Met-Leu-Phe (fMLP), wortmannin (wort) (all from Sigma–Aldrich).

Phosphoinositide standards used were: Phosphoinositides sodium salt from bovine brain (Sigma–Aldrich), PtdIns(3,4)P_2, PtdIns(3,5)P_2 and PtdIns(3,4,5)P_2 as 1,2-dioctanoyl-sn-glycero-3-phosphoinositolphosphates ammonium salt and PtdIns3*P* and PtdIns5*P* as 1-heptadecanoyl-2-(5Z,8Z,11Z,14Z-eicosatetraenoyl)-sn-glycero-3-phosphoinositolphosphates ammonium salt from Avanti Polar Lipids (Avanti Polar Lipids, Alabaster, AL).

2.6. Extraction, deacylation and sample preparation

Extraction of PIs was adapted from Ogiso et al. [27], who described a modified acidic Bligh-Dyer extraction [32] with addition of NaCl to the aqueous phase to help reducing loss of PIs. Briefly, ca. 10^6 cells were extracted with 2 ml methanol, 2 ml 1 M HCl, 0.15 ml 2 M NaCl and 2 ml chloroform (solvents from Scharlau, other reagents from Sigma–Aldrich). Methanol was supplemented with PhosSTOP (Roche, Basel, Switzerland), 1 mM NaF, 3 mM BHT and 0.5 mM phosphatidic acid (all from Sigma–Aldrich). The two phases were mixed well and centrifuged shortly for separation. The lower organic phase was removed, evaporated by nitrogen stream and transferred to deacylation. Dried samples were incubated with methylamine solution in water/methanol/n-butanol (43:46:11) at 53 °C for 50 min, all solvent was evaporated under vacuum, and then extracted with a mixture of n-butanol/petrol ether 40–60°/ethyl formiate (20:4:1) and water [33]. The water-phase was dried in vacuum and the samples were dissolved in 40 μl of solvent A for LC–MS analysis.

3. Results and discussion

3.1. Separation of phosphoinositides in order of increasing phosphorylation

Separation of anionic or phosphorylated compounds is typically achieved by ion-exchange chromatography. However, typical ion-pairing reagents are not volatile and, hence, not compatible with LC–MS. We tested several volatile and MS-compatible ion-pairing reagents, such as formic acid, ammonium formiate and N,N-dimethyl-hexylamine (DMHA) and applied them on various columns (Nucleosil C100, LiChrospher Diol and Phenomenex Aqua C18). The only acceptable separation of a phosphoinositide reference mixture was achieved on a short (75 mm) Phenomenex Aqua C18 column with the addition of DMHA. Subsequently, we tested different gradient profiles, column temperatures, pH and concentrations of DMHA to optimize separation. Column temperature had a slight impact, and the best separation was obtained at 15 °C. In contrast, pH of the mobile phase was critical. Best results were obtained around pH 7, while lower pH values lead to peak tailing and split peaks and higher pH resulted in shorter retention times. Increase of DMHA concentration from 5 to 10 mM and 20 mM did not enhance the quality of the separation. A water-acetonitrile gradient was applied and the final gradient program was 0.1–50% ACN (containing 5 mM DMHA) in 25 min, leading to the separation of a PI standard mixture shown in Fig. 2A. Peaks shown resulted from 0.1 μg of deacylated PtdIns(3,4,5)P_3 standard mixed with 4 μg of deacylated phosphoinositde extract (mixture of PtdIns, PtdIns4*P* and PtdIns(4,5)P_2).

Separation of a mixture of PIs standards was also achieved under isocratic conditions (27% ACN and 73% water) but separation of biological samples, however, could not be achieved under these conditions, probably due to interference with the biological matrix.

To test the applicability of our method to biological samples, we analysed mast cell extracts. Mast cells are known to produce large amounts of PtdIns(3,4,5)P_3 upon activation that can be provoked *in vitro* by stimulation with adenosine [30]. Murine bone marrow derived mast cells (BMMCs) were stimulated with 5 μM adenosine for 30 s, the lipids were extracted, deacylated and analysed with method 1. Our method clearly succeeded in reproducing the increased amounts of PtdIns(3,4,5)P_3 upon stimulation of mast cells with adenosine, whereas peaks of PtdIns and PtdIns*P* remained constant (Fig. 2B and C).

3.2. Positional isomer separation of phosphatidylinositol bisphosphates

To achieve separation of PtdInsP_2 positional isomers various columns were tested, including Nucleosil C100, Atlantis C18, Atlantis T3 and Phenomenex Aqua C18. The separation was only achieved on a Phenomenex Aqua column (column length 250 mm) and a water–acetonitrile gradient (containing 5 mM DMHA as ion-pair reagent) (method 2). A mixture of standards of all PtdInsP_2 isomers was separated in the elution order of PtdIns(3,4)P_2, PtdIns(4,5)P_2 and PtdIns(3,5)P_2 (Fig. 3A and B). Peaks shown resulted from 0.1 μg of deacylated PtdIns(3,5)P_2 and PtdIns(3,4)P_2 standard mixed with 4 μg of deacylated phosphoinositde extract (mixture of PtdIns, PtdIns4*P* and PtdIns(4,5)P_2). Several other solvent mixtures and addition of modifiers were tested. A separation with a different elution order (PtdIns(3,4)P_2, PtdIns(3,5)P_2 and PtdIns(4,5)P_2) was obtained with a water–methanol gradient (containing 5 mM DMHA) (method 3) (Fig. 3C and D).

Vps34 transfected HEK 293 cells under hyperosmolar stress were used as a model to test the analysis of PtdIns(3,5)P_2 from biological samples. The PI3-kinase Vps34 is known to stimulate osmotic stress related production of PtdIns(3,5)P_2 in yeast [22,28]. HEK Vps34 were incubated for 10 min in a medium supplemented with 1 M NaCl solution to induce stimulation of Vps34, and generation of PtdIns3*P* leading to production of PtdIns(3,5)P_2. As can be seen in Fig. 4A and B, the transfection with Vps34 already induced some production of PtdIns(3,5)P_2, which was then further increased upon NaCl hyperosmotic stimulation.

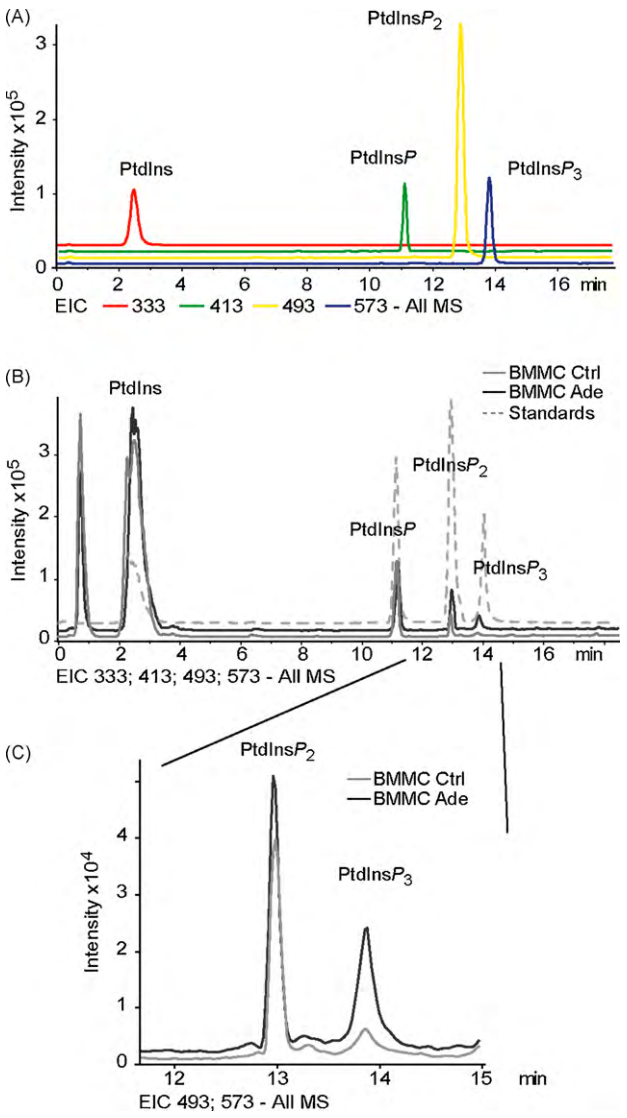

Fig. 2. (A) Extracted ion chromatogram (EIC) of deacylated phosphoinositide standards (PtdIns, PtdIns*P*, PtdInsP_2 and PtdInsP_3) mixture. (B) EIC of cell samples from 4 mio BMMC and standards. Cells were stimulated with adenosine (Ade) 5 μM for 30 s to induce production of PtdInsP_3. (C) Peaks of PtdInsP_2 and PtdInsP_3 from control cells in relation to stimulated cells, levels of PtdInsP_3 increased after stimulation with adenosine. Column: Phenomenex Aqua C18 (75 mm × 2 mm, 3 μm). Solvent A: H_2O (+5 mM DMHA), solvent B: acetonitrile (+5 mM DMHA). Gradient: 0.1% B to 50% B in 25 min.

Analysis of PtdIns(3,4)P_2 in biological samples was tested with human blood platelets. In comparison to many other cell models, platelets produce PtdIns(3,4)P_2 in relatively large amounts upon activation. PtdIns(3,4,5)P_3 is degraded to PtdIns(3,4)P_2 by the 5-phosphatase SHIP1 [34]. PtdIns(3,4)P_2 is responsible for the persistence of the signal induced by PtdIns(3,4,5)P_3 [8,35]. For detection of PtdIns(3,4)P_2, platelets were stimulated for 90 s with fMLP. This resulted in elevated amounts of PtdIns(3,4)P_2 which were not present in control cells (Fig. 4C and D).

3.3. Positional isomer separation of phosphatidylinositol monophosphates

Separation of the mono-phosphorylated isomers was only achieved with methanol–water mixtures as mobile phase (Fig. 5A and B), whereas the use of acetonitrile resulted in co-elution of PtdIns3*P* and PtdIns5*P*.

The method was applied to Vps34 transfected HEK cells as model. Vps34 is stimulated by amino acid addition through a yet unknown mechanism [36]. Starvation and subsequent amino acid supplementation stimulates Vps34 and induces the generation of PtdIns3*P* [37,38]. Thus, HEK Vps34 cells were serum and amino acid starved for 12 and 2 h, respectively. By addition of serum and amino acids, cells were stimulated for 30 min prior to extraction of lipids, deacylation and analysis. The stimulation with serum and amino acids induced production of PtdIns3*P*, which, in contrast, was inhibited by incubation with the PI3-kinase inhibitor wortmannin 15 min prior to and during stimulation with amino acids and serum (Fig. 5C and D).

3.4. Comparison with radiolabelling method

For comparison with the standard detection method of scintillation analysis, we applied radiolabelled samples to the newly developed HPLC method combined with subsequent scintillation analysis. This also gave a reconfirmation of the peaks measured with MS. The large loop size of 1 ml within the flow scintillation analyser and a flow rate of only 0.5 ml/min resulted in very broad and asymmetric peaks. Nevertheless, peaks of the major PIs could be detected (Fig. 6A and B). A radioactive labelled cell sample showed all major PI peaks (Fig. 6C) at the same retention times as when detected with MS. So did a radioactive labelled standard of PtdIns3*P* (Fig. 6C) that was clearly different to the retention time of the cellular PtdIns4*P* peak.

3.5. Discussion

The separation of PIs differing in number of phosphorylations was successfully achieved with method 1 and applied to analysis of phosphoinositides in cell samples. For analysis of PtdIns(3,4,5)P_3 this method offers a good alternative to the assay involving radiolabelling and anion-exchange HPLC. The short analysis time facilitates handling of large sample numbers.

Separation of isomers was achieved on the same column type, but of longer size and hence increased separation capacity (method 2). This assay offers new perspectives for research on phosphoinositide signalling. The low-abundant PtdInsP_2 isomers can now be separated and analysed without the need for radioactive labelling.

Replacing acetonitrile by methanol enabled the separation of PtdIns3*P*, PtdIns4*P* and PtdIns5*P* (method 3). This isomer separation has not been possible before. Also the PtdInsP_2 isomers could be separated with method 3, albeit in a different elution order when compared to method 2. This hampered a complete separation of the highly abundant PtdIns(4,5)P_2 from the trace isomer PtdIns(3,5)P_2. Therefore, analysis of the biologically important PtdIns(3,5)P_2 should be performed with method 2.

Separation of PtdInsP_2 isomers has been shown before on a cyclodextrin column [14], but co-eluting peaks of PtdIns(3,4,5)P_3 and PtdIns(3,5)P_2 limited the usefulness of the method. Also, separation of PtdIns*P* isomers was neither shown nor discussed in that publication. Compared to a cyclodextrin column the polar endcapped RP-column used here exhibits a more predictable chromatographic behaviour, offers more options for method refinement, and is widely applicable. Furthermore, robustness of the column and reproducibility and stability of separations are very high.

The methods presented here were successfully applied to relevant biological samples. The extraction procedure of PIs remains a major concern. As extensively discussed by Ogiso et al. [27], recovery rates of PIs are generally poor and decrease with increasing phosphorylation. Due to the amphiphilic properties these lipids are difficult to extract. The ionic headgroup adsorbs easily to glass surfaces, whereas the lipid moiety adsorbs to plastic. However,

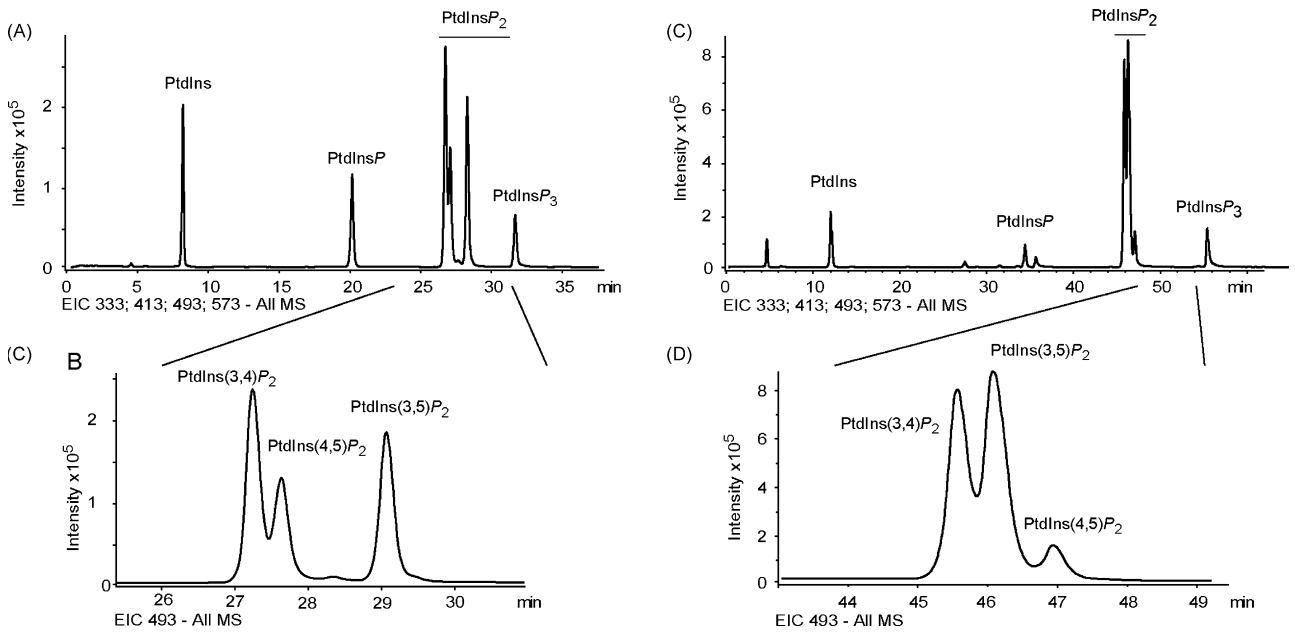

Fig. 3. (A and B) Separation of a standard mixture of PIs containing all PtdInsP_2 regioisomers. (B) Elution of the isomers of PtdInsP_2 in following order: PtdIns(3,4)P_2, PtdIns(4,5)P_2 and PtdIns(3,5)P_2. Column: Phenomenex Aqua C18 (250 mm × 4.6 mm, 5 μm). A: H_2O (+5 mM DMHA), B: acetonitrile (+5 mM DMHA). Gradient: 15% B to 35% B in 40 min. (C and D) Separation of a standard mixture of PIs containing all PtdInsP_2 regioisomers. (D) Separation of PtdInsP_2 isomers in sequence of PtdIns(3,4)P_2, PtdIns(3,5)P_2 and PtdIns(4,5)P_2. Column: Phenomenex Aqua C18 (250 mm × 4.6 mm, 5 μm). A: Methanol (+5 mM DMHA), B: acetonitrile (+5 mM DMHA). Gradient: 15% B to 50% B in 60 min.

measures can be taken to reduce loss of analytes, such as use of silanized glassware to prevent adsorption at glass surfaces [26], and lipid pre-treatment of plastic surfaces with lipids as adsorption protectants. We used plastic tubes, added lipids as adsorption protectants, and phosphatase inhibitors to prevent hydrolysis. These combined measures noticably increased extraction yields. However, further optimization is needed towards a robust and fully validated quantitative analysis of PI headgroups. This could be achieved by spiking with suitable internal standards at the beginning of the extraction procedure which ideally should be a PI with a stable-isotope labelled headgroup. However, such standards are not commercially available. An issue is the efficiency of extraction of phospholipids with different degrees of phosphorylation. There are clear indications that replacing the widely used extraction with chloroform/methanol by butanol increases the yield of highly phosphorylated PtdIns isoforms ([26], Traynor-Kaplan and

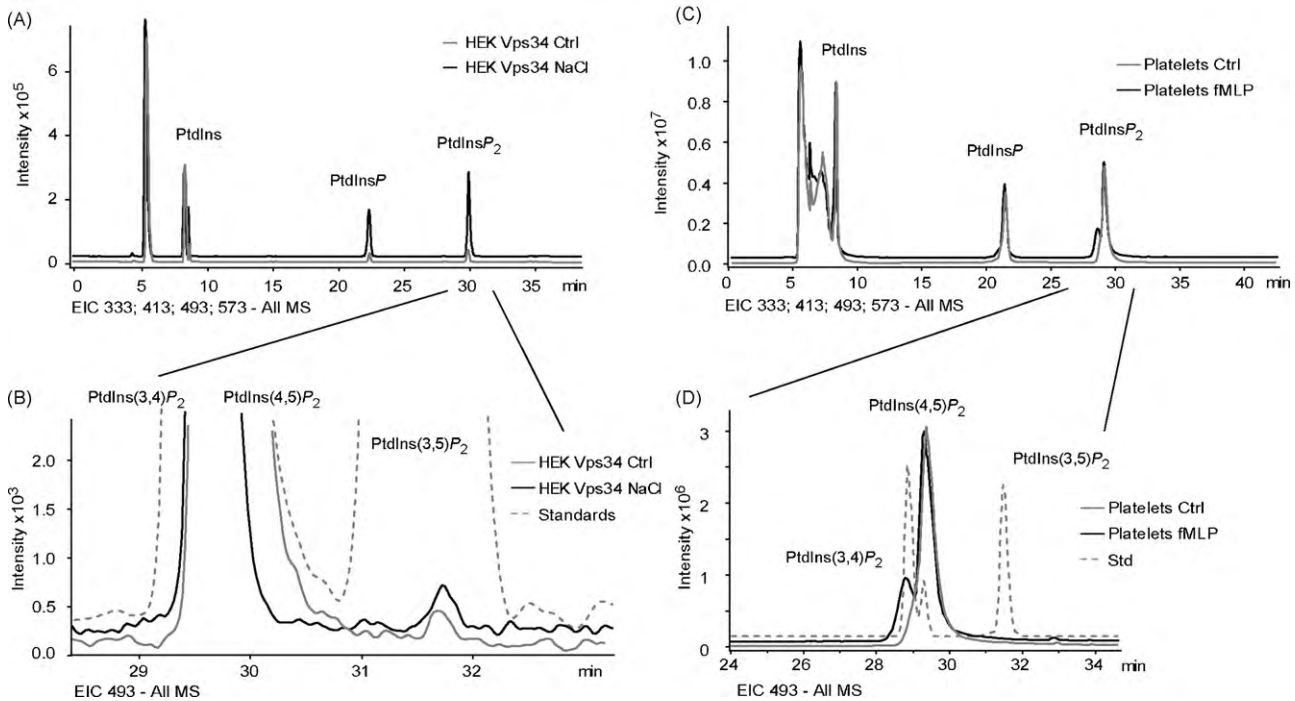

Fig. 4. Analysis of phosphoinositide cell samples. (A and B) Extracted ion chromatograms (EIC) of HEK Vps34 cell samples showed increased amounts of PtdIns(3,5)P_2, that were further increased by stimulation with 1 M NaCl for 10 min. (C and D) EIC of lipids extracted from platelets, control sample and stimulated with fMLP for 1 min. Stimulation induced generation of PtdIns(3,4)P_2 that was not present in the control sample. Column: Phenomenex Aqua C18 (250 mm × 4.6 mm, 5 μm). A: H_2O (+5 mM DMHA), B: acetonitrile (+5 mM DMHA). Gradient: 15% B to 35% B in 40 min.

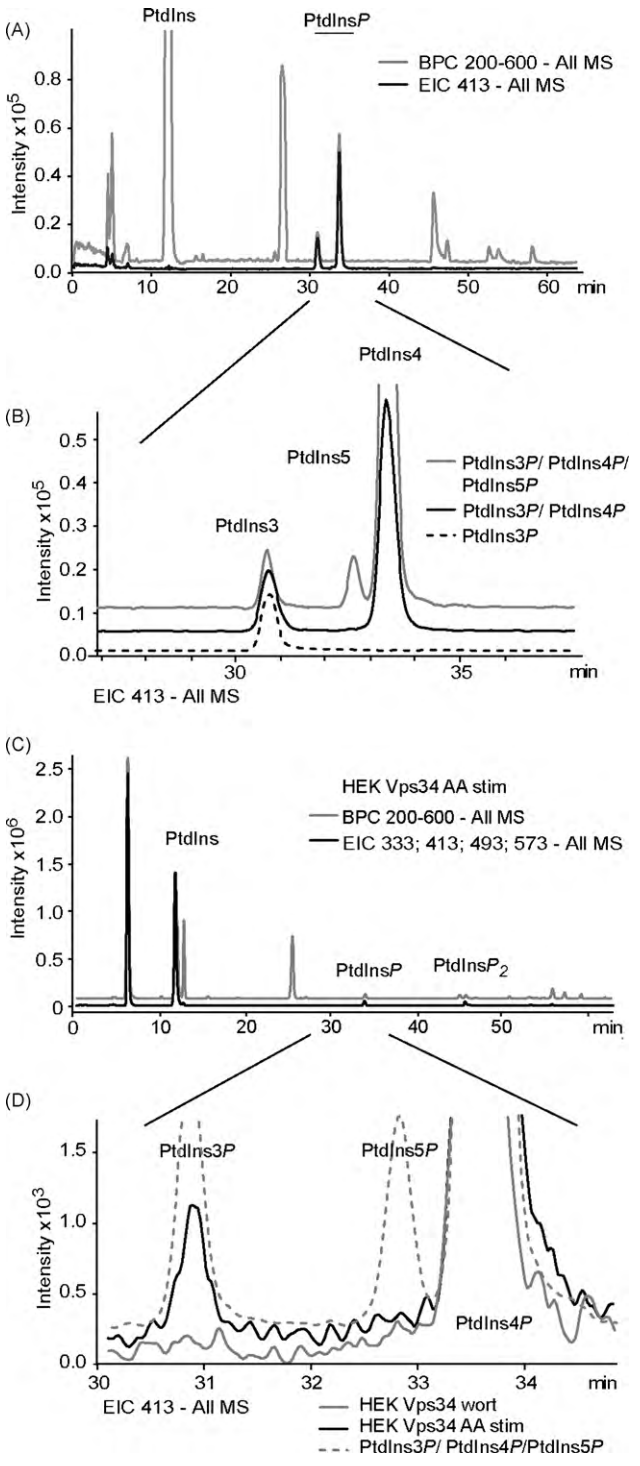

Fig. 5. (A and B) Extracted ion chromatogram (EIC) and base peak chromatogram (BPC) of mixed standards of phosphoinositide monophosphates separated according to method 3. Separation of PtdIns3*P*, PtdIns4*P* and PtdIns5*P* was achieved. (C) BPC and EIC of a HEK Vps34 cell sample. (D) EIC of HEK Vps34 cell samples that were amino acid stimulated and incubated with wortmannin 15 min prior to and during amino acid stimulation. The increase in PtdIns3*P* following amino acid starvation and subsequent stimulation was blocked by addition of wortmannin. Column: Phenomenex Aqua C18 (250 mm × 4.6 mm, 5 μm). A: H_2O (+5 mM DMHA), B: methanol (+5 mM DMHA). Gradient: 15% B to 50% B in 60 min.

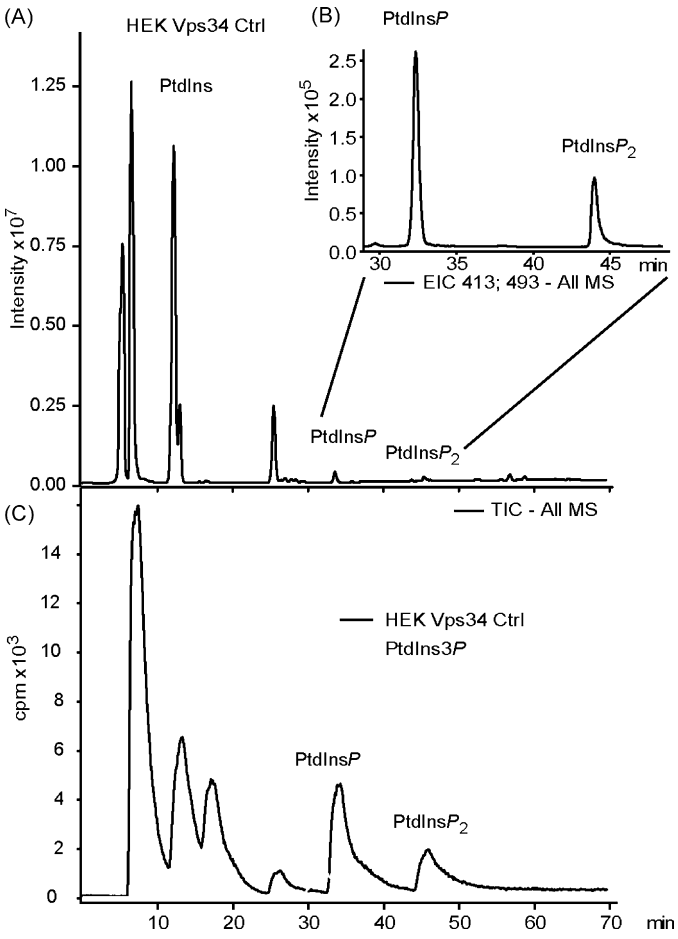

Fig. 6. (A and B) Total ion chromatogram (TIC) and extracted ion chromatogram (EIC) of a HEK Vps34 control cell sample separated according to method 3. (C) Chromatogram of parallel online flow scintillation analysis of the same HEK Vps34 cell sample and a standard of PtdIns3*P*. Column: Phenomenex Aqua C18 (250 mm × 4.6 mm, 5 μm). A: Methanol (+5 mM DMHA), B: acetonitrile (+5 mM DMHA). Gradient: 15% B to 50% B in 60 min.

Küenzi, unpublished data). Thus, a semi-quantitative analysis that compares peak intensities of highly variable (e.g. PtdIns(3,4,5)P_3) and basically unvaried (e.g. PtdIns(4,5)P_2) headgroups is currently the most suitable approach.

The direct parallel analysis with online flow scintillation and mass spectrometry showed the comparability of the two methods. The LC–MS assay presented here offers a superior approach for analysis of intracellular PI levels including differentiation of the biologically relevant PtdIns*P* and PtdInsP_2 isomers.

Further development of the method can be envisaged by a translation to UPLC, thereby taking advantage of shorter analyses and equal or superior chromatographic resolution. There is a general need for further improvement of sample workup in PI analysis, as isolation and deacylation of the PtdIns is time-consuming and difficult. Also, the issue of possible discriminatory extraction of certain PtdIns needs further investigation. Nevertheless, the LC–MS methods presented here enable simultaneous analysis of all currently known deacylated PtdIns and thus are a useful tool for cell signalling studies.

References

[1] L.C. Skwarek, G.L. Boulianne, Great expectations for PIP: phosphoinositides as regulators of signaling during development and disease, Dev. Cell 16 (2009) 12–20.
[2] M.P. Wymann, R. Schneiter, Lipid signalling in disease, Nat. Rev. Mol. Cell Biol. 9 (2008) 162–176.

[3] B. Payrastre, K. Missy, S. Giuriato, S. Bodin, M. Plantavid, M. Gratacap, Phosphoinositides: key players in cell signalling, in time and space, Cell. Signal. 13 (2001) 377–387.
[4] I.H. Batty, J. van der Kaay, A. Gray, J.F. Telfer, M.J. Dixon, C.P. Downes, The control of phosphatidylinositol 3,4-bisphosphate concentrations by activation of the Src homology 2 domain containing inositol polyphosphate 5-phosphatase 2, SHIP2, Biochem. J. 407 (2007) 255–266.
[5] M.J. Clague, S. Urbe, J. de Lartigue, Phosphoinositides and the endocytic pathway, Exp. Cell Res. 315 (2008) 1627–1631.
[6] D. Manna, A. Albanese, W.S. Park, W. Cho, Mechanistic basis of differential cellular responses of phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate-binding pleckstrin homology domains, J. Biol. Chem. 282 (2007) 32093–32105.
[7] R.H. Michell, V.L. Heath, M.A. Lemmon, S.K. Dove, Phosphatidylinositol 3,5-bisphosphate: metabolism and cellular functions, Trends Biochem. Sci. 31 (2006) 52–63.
[8] G. Di Paolo, P. De Camilli, Phosphoinositides in cell regulation and membrane dynamics, Nature 443 (2006) 651–657.
[9] J.B. Morris, K.A. Hinchliffe, A. Ciruela, A.J. Letcher, R.F. Irvine, Thrombin stimulation of platelets causes an increase in phosphatidylinositol 5-phosphate revealed by mass assay, FEBS Lett. 475 (2000) 57–60.
[10] C. Pendaries, H. Tronchere, C. Racaud-Sultan, F. Gaits-Iacovoni, S. Coronas, S. Manenti, M.P. Gratacap, M. Plantavid, B. Payrastre, Emerging roles of phosphatidylinositol monophosphates in cellular signaling and trafficking, Adv. Enzyme Regul. 45 (2005) 201–214.
[11] D. Sbrissa, O.C. Ikonomov, R. Deeb, A. Shisheva, Phosphatidylinositol 5-phosphate biosynthesis is linked to PIKfyve and is involved in osmotic response pathway in mammalian cells, J. Biol. Chem. 277 (2002) 47276–47284.
[12] A.E. Wurmser, J.D. Gary, S.D. Emr, Phosphoinositide 3-kinases and their FYVE domain-containing effectors as regulators of vacuolar/lysosomal membrane trafficking pathways, J. Biol. Chem. 274 (1999) 9129–9132.
[13] H. Guillou, L.R. Stephens, P.T. Hawkins, Quantitative measurement of phosphatidylinositol 3,4,5-trisphosphate, Methods Enzymol. 434 (2007) 117–130.
[14] P.T. Ivanova, S.B. Milne, M.O. Byrne, Y. Xiang, H.A. Brown, Glycerophospholipid identification and quantitation by electrospray ionization mass spectrometry, Methods Enzymol. 432 (2007) 21–57.
[15] K.R. Auger, L.A. Serunian, S.P. Soltoff, P. Libby, L.C. Cantley, PDGF-dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells, Cell 57 (1989) 167–175.
[16] H. Hama, J. Torabinejad, G.D. Prestwich, D.B. DeWald, Measurement and immunofluorescence of cellular phosphoinositides, Methods Mol. Biol. 284 (2004) 243–258.
[17] T.E. Rusten, H. Stenmark, Analyzing phosphoinositides and their interacting proteins, Nat. Methods 3 (2006) 251–258.
[18] S.B. Milne, P.T. Ivanova, D. DeCamp, R.C. Hsueh, H.A. Brown, A targeted mass spectrometric analysis of phosphatidylinositol phosphate species, J. Lipid Res. 46 (2005) 1796–1802.
[19] M. Pulfer, R.C. Murphy, Electrospray mass spectrometry of phospholipids, Mass Spectrom. Rev. 22 (2003) 332–364.
[20] M.R. Wenk, L. Lucast, G. Di Paolo, A.J. Romanelli, S.F. Suchy, R.L. Nussbaum, G.W. Cline, G.I. Shulman, W. McMurray, P. De Camilli, Phosphoinositide profiling in complex lipid mixtures using electrospray ionization mass spectrometry, Nat. Biotechnol. 21 (2003) 813–817.
[21] A.D. Postle, D.C. Wilton, A.N. Hunt, G.S. Attard, Probing phospholipid dynamics by electrospray ionisation mass spectrometry, Prog. Lipid Res. 46 (2007) 200–224.
[22] S.K. Dove, F.T. Cooke, M.R. Douglas, L.G. Sayers, P.J. Parker, R.H. Michell, Osmotic stress activates phosphatidylinositol-3,5-bisphosphate synthesis, Nature 390 (1997) 187–192.
[23] M. Laffargue, J.M. Ragab-Thomas, A. Ragab, J. Tuech, K. Missy, L. Monnereau, U. Blank, M. Plantavid, B. Payrastre, P. Raynal, H. Chap, Phosphoinositide 3-kinase and integrin signalling are involved in activation of bruton tyrosine kinase in thrombin-stimulated platelets, FEBS Lett. 443 (1999) 66–70.
[24] R.D. Byrne, M. Garnier-Lhomme, K. Han, M. Dowicki, N. Michael, N. Totty, V. Zhendre, A. Cho, T.R. Pettitt, M.J. Wakelam, D.L. Poccia, B. Larijani, PLCgamma is enriched on poly-phosphoinositide-rich vesicles to control nuclear envelope assembly, Cell Signal. 19 (2007) 913–922.
[25] M. Garnier-Lhomme, R.D. Byrne, T.M.C. Hobday, S. Gschmeissner, R. Woscholski, D.L. Poccia, E.J. Dufourc, B. Larijani, Nuclear envelope remnants: fluid membanes enriched in STEROLS and ploy phosphoinositides, PLOS One 4 (2009) 1–11.
[26] T.R. Pettitt, S.K. Dove, A. Lubben, S.D. Calaminus, M.J. Wakelam, Analysis of intact phosphoinositides in biological samples, J. Lipid Res. 47 (2006) 1588–1596.
[27] H. Ogiso, R. Taguchi, Reversed-phase LC/MS method for polyphosphoinositide analyses: changes in molecular species levels during epidermal growth factor activation in A431 cells, Anal. Chem. 80 (2008) 9226–9232.
[28] C. Nasuhoglu, S. Feng, J. Mao, M. Yamamoto, H.L. Yin, S. Earnest, B. Barylko, J.P. Albanesi, D.W. Hilgemann, Nonradioactive analysis of phosphatidylinositides and other anionic phospholipids by anion-exchange high-performance liquid chromatography with suppressed conductivity detection, Anal. Biochem. 301 (2002) 243–254.
[29] T. Gunnarsson, L. Ekblad, A. Karlsson, P. Michelsen, G. Odham, B. Jergil, Separation of polyphosphoinositides using normal-phase high-performance liquid chromatography and evaporative light scattering detection or electrospray mass spectrometry, Anal. Biochem. 254 (1997) 293–296.
[30] M. Laffargue, R. Calvez, P. Finan, A. Trifilieff, M. Barbier, F. Altruda, E. Hirsch, M.P. Wymann, Phosphoinositide 3-kinase gamma is an essential amplifier of mast cell function, Immunity 16 (2002) 441–451.
[31] B. Dewald, M. Baggiolini, Platelet-activating factor as a stimulus of exocytosis in human neutrophils, Biochim. Biophys. Acta 888 (1986) 42–48.
[32] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Can. J. Biochem. Physiol. 37 (1959) 911–917.
[33] N.G. Clarke, R.M. Dawson, Alkaline O leads to N-transacylation. A new method for the quantitative deacylation of phospholipids, Biochem. J. 195 (1981) 301–306.
[34] M.P. Gratacap, S. Severin, G. Chicanne, M. Plantavid, B. Payrastre, Different roles of SHIP1 according to the cell context: the example of blood platelets, Adv. Enzyme Regul. 48 (2008) 240–252.
[35] A. Sorisky, W.G. King, S.E. Rittenhouse, Accumulation of PtdIns(3,4)P2 and PtdIns(3,4,5)P3 in thrombin-stimulated platelets. Different sensitivities to $Ca2^{+}$ or functional integrin, Biochem. J. 286 (Pt 2) (1992) 581–584.
[36] P. Gulati, G. Thomas, Nutrient sensing in the mTOR/S6K1 signalling pathway, Biochem. Soc. Trans. 35 (2007) 236–238.
[37] T. Nobukuni, M. Joaquin, M. Roccio, S.G. Dann, S.Y. Kim, P. Gulati, M.P. Byfield, J.M. Backer, F. Natt, J.L. Bos, F.J. Zwartkruis, G. Thomas, Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase, Proc. Natl. Acad. Sci. U.S.A. 102 (2005) 14238–14243.
[38] M.P. Byfield, J.T. Murray, J.M. Backer, hVps34 is a nutrient-regulated lipid kinase required for activation of p70 S6 kinase, J. Biol. Chem. 280 (2005) 33076–33082.