Absolute quantitation of inositol pyrophosphates by capillary electrophoresis electrospray ionization mass spectrometry

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SUMMARY

We describe a procedure for capillary electrophoresis electrospray ionization mass spectrometry for the absolute quantitation of inositol pyrophosphates from mammalian cell extracts.

ABSTRACT

Inositol pyrophosphates (PP-InsPs) are an important group of intracellular signaling molecules. Derived from inositol phosphates (InsPs), these molecules feature the presence of at least one energetic pyrophosphate moiety on the myo-inositol ring. They exist ubiquitously in eukaryotes and operate as metabolic messengers surveying phosphate homeostasis, insulin sensitivity, and cellular energy charge. Owing to the absence of a chromophore in these metabolites, a very high charge density, and low abundance, their analysis requires radioactive tracer, and thus it is convoluted and expensive. Here, the study presents a detailed protocol to perform absolute and high throughput quantitation of inositol pyrophosphates from mammalian cells by capillary electrophoresis electrospray ionization mass spectrometry (CE-ESI-MS). This method enables the sensitive profiling of all biologically relevant PP-InsPs species in mammalian cells, enabling baseline separation of regioisomers. Absolute cellular concentrations of PP-InsPs, including minor isomers, and monitoring of their temporal changes in HCT116 cells under several experimental conditions are presented.

INTRODUCTION

Since the initial discovery of *myo*-inositol pyrophosphates (PP-InsPs) in 1993[1,2], significant progress has been made to elucidate their biosynthesis, turnover, and functions[3]. Inositol pyrophosphates ubiquitously occur in eukaryotic cells[4] and serve as metabolic signalling molecules critically involved in e.g. phosphate homeostasis[5,6], insulin sensitivity[7], calcium oscillations[8,9], vesicular trafficking[10], apoptosis[11], DNA repair[12], immune signalling[13], and others. The plethora of important processes under the control of inositol pyrophosphates calls for a deeper understanding of their cellular abundance, fluctuation, and localisation.

Although Inositolphosphates (InsPs) and PP-InsPs attracted attention across disciplines, the analysis of their abundance is routinely performed using a method developed during the '80s, consisting in labelling cells with tritiated inositol, resolving the extracted PP-InsPs by strong anion exchange chromatography Sax-HPLC with subsequent scintillation counting. Newer methods based on mass spectrometry still face significant challenges: inositol pyrophosphates with up to eight phosphate units harbour phosphate esters and anhydrides leading to significant negative charge and potential phosphate loss during ionisation. There are four major types of PP-InsPs found in mammals (Figure 1): 1,5-$(PP)_2$-$InsP_4$ (or 1,5-$InsP_8$), 5-PP-$InsP_5$ (or 5-$InsP_7$), 1-PP-$InsP_5$ (or 1-$InsP_7$) and 5-PP-Ins(1,3,4,6)P_4 (or 5-PP-$InsP_4$)[3,14]. The physiological levels of PP-InsPs are typically in the nano- to low micromolar range with 5-PP-$InsP_5$ as the most abundant with cellular concentrations of 0.5 – 5 µM. 1,5-$(PP)_2$-$InsP_4$ and 1-PP-$InsP_5$ are believed to be up to around 10 % of the 5-PP-$InsP_5$ pool and remain difficult to trace in many cells[15]. 5-PP-$InsP_4$ with a free OH group is even lower in abundance and usually only becomes detectable when phosphate hydrolases are inhibited with sodium fluoride (NaF)[16].

The high charge density of PP-InsPs makes their separation difficult, and the occurrence of PP-InsP regioisomers further complicates these efforts. As a result, most experiments relied on quantitation by metabolic radioactive labeling of cells using [^{3}H]-inositol, as background from the matrix is excluded and high sensitivity is achieved[17,18]. However, this method is costly, time-consuming, and does not allow to properly distinguish related PP-InsP regioisomers. Moreover, [^{3}H]-inositol labeling does not account for endogenous inositol synthesis from glucose. A polyacrylamide gel electrophoresis (PAGE)-based method is a widely applied inexpensive alternative but limited in its sensitivity[19,20,21,22]. Other approaches avoiding radiolabeling have been published, including ion chromatography followed by post-column derivatization UV-detection[23], hydrophilic interaction chromatography (HILIC)[24], or weak anion exchange (WAX) coupled with mass spectrometry (MS)[25]. However, they are not (yet) on par with the classic [^{3}H]-inositol SAX-HPLC protocol.

Recently, capillary electrophoresis electrospray ionization mass spectrometry (CE-ESI-MS) was introduced as a transformative strategy for the analysis of InsPs and PP-InsPs metabolism, meeting all requirements discussed above[16]. Combined with current state-of-the-art InsP extraction by perchloric acid followed by enrichment with titanium dioxide beads[26], CE-ESI-MS succeeded in every organism tested so far, from yeast to plants and mammals. Simultaneous profiling of InsPs and PP-InsPs, including all possible regioisomers, was easily achieved. Stable isotope-labeled (SIL) internal standards enabled a rapid and precise absolute quantitation, irrespective of matrix

effects. Because MS can capture isotopic mass differences, CE-ESI-MS can also be applied to study compartmentalized cellular synthesis pathways of InsPs and PP-InsPs, e.g., by feeding cells with [$^{13}C_6$]-*myo*-inositol or [$^{13}C_6$]-D-glucose.

Described here is a detailed step-by-step protocol for the absolute quantitation of PP-InsPs and InsPs from mammalian cells by CE-ESI-MS. Apart from the major 5-PP-InsP_5 isomer, 1,5-$(PP)_2$-InsP_4 and 1-PP-InsP_5 are also quantified in this study, despite their lower abundance. Two HCT116 cell lines from different laboratories (NIH, UCL) are studied, and it is validated that HCT116^{UCL} cells contain 7-fold higher levels of 1,5-$(PP)_2$-InsP_4 than found in HCT116^{NIH}, while 5-PP-InsP_5 concentrations are comparable. In addition, 1-PP-InsP_5 synthesis in HCT116^{UCL} is not significantly increased. Also, the increase of PP-InsP levels by blocking their dephosphorylation using sodium fluoride is studied quantitatively.

PROTOCOL

1. Setting up the CE-ESI-MS system

    1.1 Set up a CE-ESI-MS system consisting of a commercial CE system (e.g. Agilent 7100) and a triple quadrupole tandem mass spectrometer (e.g. Agilent QQQ 6495c), equipped with an Agilent Jet Stream (AJS) electrospray ionization (ESI) source. A CE-ESI-MS sprayer kit and an isocratic LC pump (e.g. Agilent 1200) are requisite.

    1.2 Connect the sheath flow 1:100 splitter (included in the Agilent CE-MS sprayer kit) and the isocratic LC pump outlet.

    1.3 Make sure that the CE system inlet vial is at the same height to the sprayer tip of mass analyzer.

    1.4 Utilize Agilent MassHunter Workstation (Version 10.1) or comparable MS software to control the entire system, and for data acquisition and analysis.

2. Preparing buffer, capillary and CE-MS system

    2.1 Prepare CE running buffer: 40 mM ammonium acetate adjusted to pH 9.0 with ammonium hydroxide. A 250 mL volumetric flask is recommended. Filter the 250 mL buffer with 0.2 µm pore-sized membrane filters. This buffer can be kept at room temperature for 2-3 weeks or alternatively several months in a fridge. Ultra-pure deionized water and MS grade reagents are essential.

    2.2 Prepare sheath liquid: Mix 100 mL ultra-pure water and 100 mL LC-MS grade *iso*-propanol in a 250 mL bottle. Change the sheath liquid at least once a week. Add mass reference into the sheath liquid when employing a high-resolution mass spectrometer.

    2.3 Install sheath liquid: Purge at 5 mL/min for 5 mins and set flow rate at 1 mL/min (10 µL/min into the CE-MS sprayer). The pump pressure will be at ca 180 bar. Make sure that the recycle tubing connects back into the sheath liquid bottle to reuse the solvent.

    2.4 Prepare capillary: CE-MS capillary (50µm i.d. 365µM o.d. with a length of 125 cm) with

UV detection window can be purchased. Much cheaper bar fused silica capillaries can be also be obtained from specialized distributors, for example, CS-Chromatographie Service GmbH (Germany). Cut a capillary with a length of 100 cm. Properly cut both capillary ends with a capillary column cutter with a rotating diamond blade and remove 2-3 cm polyimide coating on both ends with a lighter. Clean the capillary surface with isopropanol.

2.5 Install capillary: Match the capillary into the CE-MS cassette. Click on the Change Cassette button and install the cassette into the CE device. The inlet end of the capillary is around 2 mm lower than the electrode. Ensure that the inlet end is lower than the sample's surface during the injection process.

2.6 Activate capillary: Prior to first use, flush the capillary with 1 M NaOH, followed by water for 10 min, and CE running buffer for 15 min.

2.7 Insert the capillary end into the CE-MS sprayer: Gently put the capillary into the CE-MS sprayer and ensure that the capillary end protrudes approximately 0.1 mm out of the sprayer tip. The precise adjustment of the capillary outlet end must be done with a magnifying glass and the adjustment screw in the sprayer. Insert the sprayer back into the ion source, and avoid touching the adjustment screw. The MS should be on Standby mode when performing this operation.

2.8 Check ESI spray: Check the stability of ESI sprayer under full scan mode. The fluctuation of total ion electropherograms should be within 5%.

2.9 Perform a test run with InsP standards: A mixture of 2 µM $InsP_3$-$InsP_8$ standards (adjusted by quantitative ^{31}P NMR[15,27]) are employed for test runs with an injection at 50 mbar for 10 s (10 nL). Detailed ESI and MS parameters are set as shown in Table 1. CE current is ca 26 µA. Peak width is around 0.5 min. Ensure that the signal-to-noise ratio reaches at least 400.

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3. Extraction of soluble inositol phosphates from mammalian cells

$HCT116^{NIH}$ were a kind gift from Stephen Shears[28]. $HCT116^{UCL}$ are from Saiardi's Lab[26].

3.1 Seeding cells

3.1.1 Culture $HCT116^{NIH}$ or $HCT116^{UCL}$ cells in T75 flasks at 37 °C in a 5% high humidity CO_2 atmosphere (further referred to as standard conditions) in Dulbecco's modified eagle medium (DMEM) supplemented with 10 % fetal bovine serum (FBS).

3.1.2 Wash $HCT116^{NIH}$ and $HCT116^{UCL}$ stock cultures with phosphate buffered saline (PBS) (5 mL) and incubate the cells with trypsin-ethylenediamine tetraacetic acid (EDTA) (3 mL, 0.25 %) under standard conditions until they are completely detached. Quench trypsin activity by adding medium and collect the cells into a centrifuge tube and centrifuge (200 g, 3 min).

3.1.3 Remove the supernatant and resuspend the cells in medium (10 mL). Count the cells and determine the viability via trypan blue exclusion.

3.1.4 Seed the cells (6 million HCT116 cells per assay) into a 150 mm dish and adjust 20 mL of cell culture medium in total. Premix the medium and the cells in a centrifuge tube prior to seeding in order to achieve an equal distribution of the cells in the dish. Prepare a parallel dish when normalization by cell number is required.

3.1.5 Culture the cells under standard conditions for 72 h. The cells will reach about 80-90 % confluence.

3.2 Modulation of inositol phosphate levels with NaF and cell harvesting

3.2.1 NaF treatment: Add NaF (10 mM) 1 h before harvest into the medium. Mix the medium by swirling the plate/pipetting and incubate the cells for 60 min under standard conditions.

3.2.2 After NaF treatment, remove the medium from the cells and place the cells on ice.

3.2.3 Wash the cells twice with PBS (5 mL, 4 °C) and remove the PBS completely from the dish.

3.2.4 Add perchloric acid (PA) (1 mL, 1 M, 4 °C). Ensure that the whole surface is covered with PA (the cells will turn white as proteins precipitate). Incubate the cells for 10 min on a tilt table at 4 °C.

3.2.5 Collect PA into a centrifuge tube and remove contaminating debris by centrifugation (17.000 g, 5 min, 4 °C). Add the supernatant to the prepared TiO_2 beads for the pulldown of InsPs.

3.2.6 Wash the post-extraction dish twice with PBS (5 mL, r.t.) for deacidification; remove PBS completely from the dish.

3.2.7 Solubilize proteins on the plate via addition of cell lysis buffer (1.5 mL, r.t.; 0.1 % sodium dodecyl sulfate [SDS] in 0.1 M NaOH). Incubate the dish for 15 min on a tilt table at r.t. Transfer the cell lysate into a centrifuge tube and centrifuge (17.000 g, 5 min, 4 °C). Store the supernatant at -80 °C until the protein concentration is determined via the DC protein assay using bovine serum albumin as calibration standard (usually, one 150 mm dish contains around 10 mg of proteins).

3.2.8 Determination of cell numbers from parallel dishes: Harvest the cells of the parallel dish via trypsin as described in section 3.1.2 (use 5 mL trypsin-EDTA for the 150 mm dish) and remove the medium. Resuspend the cell pellet in PBS (5 mL), mix properly, and count the cells. Perform this step right before harvest via direct quenching to obtain representative cell counts. Additionally, measure the volume of the cells with an appropriate method (e. g. with a multisizer machine).

3.3 TiO_2 enrichment of inositol phosphates

To avoid acidic decomposition of phosphorylated compounds, all steps of the enrichment until elution need to be performed on ice and all reagents need to be cooled to 4 °C. The time for the extraction should be kept to a minimum (1.5-2 h). All of the extraction steps are performed with 1 M PA.

3.3.1 Preparation of beads: Wash TiO_2 beads (5 mg per sample) with ddH_2O (1 mL) and centrifuge (3.500 g, 1 min, 4 °C). Remove ddH_2O and wash the beads with PA (1 mL). Remove PA by centrifugation (3.500 g, 1 min, 4 °C). Resuspend the beads in PA (50 µL per sample).

3.3.2 Add the supernatant containing phosphorylated compounds (compare section 3.2, step 3.2.6) to the bead suspension, vortex and then rotate the sample for 20 min at 4 °C.

3.3.3 Centrifuge the sample (3.500g, 1 min, 4 °C) and discard the supernatant. Wash the beads with PA (500 µL) and centrifuge (3.500g, 1 min, 4 °C). Discard the supernatant and repeat the washing step.

3.3.4 Add NH_4OH (200 µL, 3 %) to the beads and resuspend. Rotate the sample for 5 min at r.t.

3.3.5 Centrifuge the sample (3500 g, 1 min) and transfer the supernatant into a new centrifuge tube.

3.3.6 Repeat elution steps 3.3.4 and 3.3.5 and combine the eluents. The beads can be discarded.

3.3.7 Centrifuge the combined eluents (17.000 g, 1 min, 4 °C) to remove any insoluble residues.

3.3.8 Completely dry the supernatant under vacuum evaporation (70 min, 60 °C, V-AQ). Add ddH_2O (50 µL) to the dried extracts containing InsPs. Vortex mix the sample until completely dissolved. Store the sample at -20 °C until CE-ESI-MS analysis.

4. Performing the CE-ESI-MS runs

4.1 Preparing a mixture of internal standards containing 40 µM [$^{13}C_6$]1,5-$(PP)_2$-$InsP_4$, 80 µM [$^{13}C_6$]5-PP-$InsP_5$, 80 µM [$^{13}C_6$]1-PP-$InsP_5$, 400 µM [$^{13}C_6$]$InsP_6$ and 400 µM [$^{13}C_6$]Ins(1,3,4,5,6)P_5. All above SIL internal standards (IS) with purities higher than 96% were synthesized and provided by Fiedler group[15,27]. Concentrations of SIL IS solutions were determined by quantitative ^{31}P and ^{1}H NMR against a certified reference standard (phosphoacetic acid).

4.2 Mix 10 µL sample with 0.5 µL internal standards mixture in CE sample vial. 2 µM [$^{13}C_6$]1,5-$(PP)_2$-$InsP_4$, 4 µM [$^{13}C_6$]5-PP-$InsP_5$, 4 µM [$^{13}C_6$]1-PP-$InsP_5$, 20 µM [$^{13}C_6$]$InsP_6$ and 20 µM [$^{13}C_6$]Ins(1,3,4,5,6)P_5 are the final concentrations inside samples.

4.3 When using replenishment system, put the prepared 250 mL CE running buffer into the electrolyte bottle, click clean tubes. Keep the replenishment needle in a water vial.

4.4 Set ESI and MS parameters as shown in Table 1.

4.5 Perform a run for the InsP extracts and check the result (Figure 2). Run a sequence when there are more samples.

4.6 Standby the MS after measurements. Do not turn off the LC pump. The flow of sheath liquid protects the sprayer needle.

5. Data analysis

5.1 Open Agilent MassHunter Quantitative Analysis (for QQQ), create a batch for all samples.

5.2 Create new Method from acquired MRM data. Set the internal standards as ISTD. Check MRM compound setup, retention time setup, ISTD setup, concentration setup and qualifier setup. Pass the validation and exit to apply the method to current batch.

5.3 Check if each peak in the batch are properly integrated, otherwise manually integrate the peak.

5.4 Export the results into a excel file. Quantitation of inositol (pyro)phosphates are performed by comparing analyte peak response with the respective peak response of SIL IS with known concentrations.

5.5 With the measured concentration in the InsP extract solution and its volume, absolute amounts can be calculated. Further normalize the amount by cell counts or protein content. Cellular concentration is calculated based on cell counts and average cell volume of HCT116 (1.68 fL).

REPRESENTATIVE RESULTS

The results shown here aim to illustrate the potential of CE-ESI-MS analysis. The reported figures are descriptive of technically flawless CE-ESI-MS run. Firstly, a mixture of inositol pyrophosphate standards (Figure 1) and a mammalian cell extract (Figure 2) are presented. Secondly, a comparison of two HCT116 cell lines (Figure 3) and NaF-treated HCT116 (Figure 4) cells are provided.

Extracted ion electropherograms (EIEs) of inositol (pyro)phosphate standards at a concentration of 2 μM are shown in Figure 1. Metabolism of inositol pyrophosphates in mammals with their simplified structures is inserted. The four inositol pyrophosphates in mammals, 1,5-$(PP)_2$-$InsP_4$, 5-PP-$InsP_5$, 1-PP-$InsP_5$ and 5-PP-Ins(1,3,4,6)P_4 are well distinguished using the described method.

A CE-ESI-MS run of $HCT116^{UCL}$ is depicted in Figure 2. With the aid of stable isotope labeled (SIL) internal standards, an absolute quantitation can be readily achieved by comparing the signal response with the spiked-in SIL of known concentration. The integrated EIEs of the inositol

phosphate from $InsP_5$- to $(PP)_2$-$InsP_4$) and un-integrated EIEs of their isotopic patterns are displayed. RSDs of all analytes from six technical repeats are within 4%. With the measured concentration and the volume of extracts, the amount of analytes can be calculated. With the cell counts and cell volume, or protein content, absolute cellular concentration (µM) or amount normalized by protein content (pmol/mg protein) are commonly the final outcomes of such an analysis.

According to an earlier study, two batches of diverged HCT116 cells have variation of $InsP_8$ levels, $HCT116^{UCL}$ cells contain 6-fold higher levels of $InsP_8$ than $HCT116^{NIH}$ cells[29]. With the CE-MS method, 1,5-$(PP)_2$-$InsP_4$ in $HCT116^{NIH}$ could be easily quantified (Figure 3), and $HCT116^{UCL}$ cells contain 7-fold higher levels of $InsP_8$ than in $HCT116^{NIH}$. In addition, the higher accumulation of 1,5-$(PP)_2$-$InsP_4$ in $HCT116^{UCL}$ cells is not accompanied by a significantly increased 1-PP-$InsP_5$ synthesis, which is now quantitatively shown in Figure 3.

Levels of PP-InsPs increase by blocking their dephosphorylation using sodium fluoride. CE-ESI-MS analysis of NaF-treated $HCT116^{NIH}$ demonstrated the expected 5-PP-$InsP_5$ elevation with concomitant reduction in $InsP_6$ and an appearance of 5-PP-Ins(1,3,4,6)P_4 (Figure 4). Besides, the elevation of $InsP_8$ levels is noticeable, while 1-PP-$InsP_5$ decreases to some degree. 1-PP-$InsP_5$ is not completely absent in NaF-treated $HCT116^{NIH}$, but mostly either under limit of detection or quantitation.

FIGURE AND TABLE LEGENDS

Figure 1: **Typical extracted ion electropherograms (EIEs) of inositol (pyro)phosphate standards in CE-ESI-MS analysis using the described protocol.** Concentration of each analyte is 2 µM. Injected sample volume is ca 10 nL with an injection at 50 mbar for 10 s. Inserts show metabolism of inositol pyrophosphates in mammals. IPPK: inositol pentakisphosphate 2-kinase, IP6K: inositol hexakisphosphate kinase, PPIP5K: diphosphoinositol pentakisphosphate kinase, DIPP1: diphosphoinositolpolyphosphate phosphohydrolase 1.

Figure 2: **Representative InsP profile of $HCT116^{UCL}$ cells.** (A) EIEs of the main inositol (pyro)phosphates in $HCT116^{NIH}$ and spiked SIL ISs 2 µM [$^{13}C_6$]1,5-$(PP)_2$-$InsP_4$ **(1)**, 4 µM [$^{13}C_6$]5-PP-$InsP_5$ **(2)**, 4 µM [$^{13}C_6$]1-PP-$InsP_5$**(3)**, 20 µM [$^{13}C_6$]$InsP_6$ **(4)** and 20 µM [$^{13}C_6$]Ins(1,3,4,5,6)P_5**(5)** . Inserts show six technical repeats of InsP analysis by CE-ESI-MS, data are presented as means ± SD. (B) Cellular concentration of PP-InsPs and InsPs in human cell lines $HCT116^{UCL}$ and (C) PP-InsPs and InsPs amount normalized by protein content. Data are means ± SEM from three independent experiments.

Figure 3: **Variation in $InsP_8$ levels between two diverged HCT116 cells.** (A) EIEs of inositol pyrophosphate in $HCT116^{UCL}$ and $HCT116^{NIH}$. $InsP_8$ in $HCT116^{UCL}$ is markedly more abundant than in $HCT116^{NIH}$. (B) Ratio of inositol pyrophosphate to $InsP_6$ (%) in both HCT116 cells. $HCT116^{UCL}$ cells contain 7-fold higher levels of $InsP_8$ as compared to in $HCT116^{NIH}$, while the 5-PP-$InsP_5$ levels are equal. Data are means ± SEM from three independent experiments.

Figure 4: I**nositol (pyro)phosphate levels in HCT116^{NIH} cells, with NaF treatment**. (A) EIEs of inositol (pyro)phosphate in HCT116^{NIH} with sodium fluoride treatment (NaF, 10 mM). Levels of inositol pyrophosphate including 1,5-$(PP)_2$-InsP_4, 5-PP-InsP_5 and 5-PP-Ins(1,3,4,6)P_4 increase via blocking their dephosphorylation using NaF. (B)Inositol (pyro)phosphate levels (amounts are normalized by protein content) in untreated and NaF-treated HCT116^{NIH} cells. Data are means ± SEM from three independent experiments.

Table 1: CE-ESI-MS parameter settings. Source parameter and iFunnel parameters are optimized by Source and iFunnel Optimizer. MSM parameter setting for inositol (pyro)phosphates are optimized by MassHunter Optimizer.

DISCUSSION

Here we present a practical and sensitive method for the quantitation of highly charged inositol pyrophosphates in mammalian cells. Combining this analysis approach with current state-of-the-art InsP extraction with perchloric acid followed by enrichment with TiO_2, CE-ESI-MS analysis has unprecedented advantages. With regards to its throughput, sensitivity, stability, absolute quantitation, isomer identification and matrix in-dependence, this method stands out compared to other approaches. Here we only describe the protocol for mammalian cells, but indeed this strategy succeeds in many different samples (e.g. yeast, plants, parasites, mouse tissues etc.).

In our laboratory, the CE-ESI-MS device runs smoothly and can accommodate around 200 samples every week. Unlike HPLC though, CE has been regarded a method for experts and specialized persons for a long time, which restricted its market and limited its application. Thus, a CE-ESI-MS device is usually absent in analytical faculties. People who wants to carry out CE-ESI-MS analysis probably lack CE experience and will spend more time on trouble shooting. Here, we therefore emphasize the critical steps. First and foremost is the quality of the capillary cut. Sensitivity and stability of ESI spray mostly rely on a first-class capillary cut. Secondly, the capillary outlet end should be exactly 0.1 mm out of the sprayer tip. Sprayer needle and the CE capillary should be in axial direction. The quality of the ESI spray is critical for quantitation, technical runs should be performed to evaluate the repeatability.

With the described protocol, limit of quantitation (LOQ) for PP-InsPs is 40 nM with an injection at 50 mbar for 10s (10 nL). There are several approaches to further increase the method sensitivity. Firstly, an injection at 100 mbar for 20s (40 nL) will still result in a good peak shape and sufficient resolution for regioisomers 5-PP-InsP_5 and 1-PP-InsP_5. Secondly, InsP extracts can be dissolved in a smaller amount of water. Thirdly, the dwell time could be increased when using less MRM transitions for quantitation.

The CE running buffer with pH 9 provides the best resolution between InsP_6-InsP_8. When increasing pH to 9.7, the resolution among InsP_3-InsP_6 will significantly improve. Due to the excellent resolution, a shorter capillary length of 60 cm is recommended for further increasing the throughput. According to different research demands, modifications of this method can

further facilitate InsPs and PP-InsPs analysis. Therefore, the described CE-ESI-MS protocols has the potential of opening novel research avenues into this multifaceted family of signaling molecules.

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DISCLOSURES

The authors declare no competing interests.

METERIALS

Name	Company	Catalog Number	Comments
Materials			
1.5 mL microcentrifuge tubes	Greiner Bio-One	616201	-
15 cm tissue culture dishes	Thermo Fisher	168381	-
2.0 mL microcentrifuge tubes	Greiner Bio-One	623201	-
50 mL centrifuge tubes	Greiner Bio-One	227261	-
96-well plates	Thermo Fisher	260836	for the DC protein assay
CE fused silica capillary	CS Chromatographie	105180	50 µM i.d. 360 µM o.d.
Pipette tips	Starlab	I1054-0001, S1111-6701, S1113-1700, S1111-3700	10 mL, 1000 µL, 200 µL, 10 µL pipette tips
Serological pipets	TPP	94550, 94525, 94010, 94005	50 mL, 25, mL, 10 mL, 5 mL serological pipettes
T75 flasks	TPP	90076	-
Chemicals and Reagents			
NaOH	AppliChem	A6829,0500	sodium hydroxide pellets for molecular biology, for

			preparation of cell lysis buffer
0.25% trypsin-EDTA	Gibco	25200056	-
Ammonium acetate	Thermo Fisher	1677373	HPLC grade
BSA	Thermo Fisher	23209	albumin standard (2.0 mg/mL) for standard curve preparation
DC protein assay	Biorad	5000116	DC protein assay reagents package
DMEM	Gibco	41966029	high glucose, pyruvate
FBS	Gibco	10270106, 10500064 (heat inactivated)	10270106 for $HCT116^{UCL}$, 10500064 for $HCT116^{NIH}$
Isopropanol	Carl Roth	AE73.2	99.95% LC-MS grade
NH_4OH, 10%	Carl Roth	6756.1	for preparation of 3% NH_4OH
PBS	Gibco	10010015	-
Perchloric acid, 70%	Carl Roth	9216.1	for preparation of 1 M perchloric acid
SDS	SERVA	20760.02	for preparation of cell lysis buffer
Sodium fluoride	Sigma Aldrich	S7920	-
TiO_2 beads	GL Sciences	5020-75000	5 µm particle size
Trypan blue solution	Gibco	15250061	trypan blue stain (0.4%)
Ultrapure (Type 1) water	Milli-Q	ZRQSVP3WW	model: Direct-Q 3 UV Water Purification System
Equipment			
Analytical balance	Mettler Toledo	30105893	model: XPE26; for weighing of beads (5-6 mg per sample)
Automated cell counter	Logos Biosystems	L40002	model: LUNA-II Automated Cell Counter
Benchtop centrifuge	Hettich	1401	model: UNIVERSAL 320
Benchtop centrifuge with cooling	VWR	521-1647P	model: Microstar 17R
CE system	Agilent	G7100A	-
CE/MS Adapter Kit	Agilent	G1603A	-
CE/MS Sprayer Kit	Agilent	G1607A	-
Cell counting slides	Logos Biosystems	L12001	LUNA Cell Counting Slides
Centrifugal evaporator	Eppendorf	5305000304	model: Concentrator plus

			complete system
ESI source	Agilent	AJS ESI	-
Super Support Film	Nisshin EM Co. Ltd, Tokyo	647	
Humidified incubator	Binder	9040-0088	model: CB E6.1, for cultivation of mammalian cells
Ice box	-	-	should provide enough space for samples, dishes, etc.
Isocratic LC system	Agilent	G7110B 1260 Iso Pump	model: Infinity II Quaternary system
MSD	Agilent	G6495C	triple quadrupole
Multiplate reader	Tecan	30086375	model: SPARK 10 M
Pipette filler	Thermo Fisher	10072332	for serological pipettes
Pipettes	Brand	705884, 705880, 705878, 705872, 705870	various pipettes
Rotator	Labnet	H5500	model: Mini LabRoller Rotator
Shortix capillary column cutter	SGT	S0020	-
Test tube shaker (vortex mixer)	Carl Roth	HXH6.1	model: Rotilabo-Mini Vortex
Tilt table	Labnet	S0600	model: EDURO MiniMix Nutating Mixer
Water bath	Thermo Fisher	FSGPD05	model: Isotemp GPD 05
Software			
MassHunter Workstation	Agilent	Version 10.1	-
MassHunter Workstation LC/MS Data Acquisition	Agilent	Version 10.1	-
MassHunter Workstation Optimizer	Agilent	Version 10.1	-
MassHunter Workstation Qualitative Analysis	Agilent	Version 10.0	-
QQQ Quantitaion	Agilent	Version 10.1	-

Analysis

References

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	Parameter	Setting or Values
CE	Inlet Home	Vial 1
	Outlet Home	Not important
	Cassette Temperature	25.0 °C
	Replenishment	Replenish Vial, Vial 1 to 1.4 cm
	Precondition	Flush, 500s (Inlet Home Vial)
	Injection	1. Apply pressure, 50 mbar for 10 s (Injection Vial) 2. Apply Pressure, 50 mbar for 5 s (Inlet Home Vial)
	Timetable	0.3 min, Change Voltage, 30 kV
	Stoptime	30 min
Iso Pump	Flow	1 mL/min
ESI Source	Gas Temp	150 °C
	Gas Flow	11 L/min
	Nebulizer	8 psi
	Sheath Gas Temp	175 °C
	Sheath Gas Flow	8 L/min
	Capillary	2000 V (Negative)
	Nozzle Voltage	2000 V (Negative)
QQQ	Scan Type	MRM
	Ion Funnel Parameters	Neg High Pressure RF, 70 V Neg Low Pressure RF, 40 V
	MS Scan	MRM (see below)

Compound Name	Precursor Ion	Product Ion	dwell	Frag (V)	CE (V)	Cell Acc (V)	Polarity
$[^{13}C_6]InsP_8$	411.9	362.9[a]	50	166	10	1	Negative
$[^{13}C_6]InsP_8$	411.9	78.9	50	166	42	4	Negative
$InsP_8$	408.9	359.9[a]	50	166	10	1	Negative
$InsP_8$	408.9	78.9	50	166	42	4	Negative
$[^{13}C_6]InsP_7$	371.9	322.9[a]	50	166	10	3	Negative
$[^{13}C_6]InsP_7$	371.9	78.9	50	166	38	3	Negative
$InsP_7$	368.9	319.9[a]	50	166	10	3	Negative
$InsP_7$	368.9	78.9	50	166	38	3	Negative
$[^{13}C_6]InsP_6$	331.9	487	50	166	10	1	Negative
$[^{13}C_6]InsP_6$	331.9	78.9[a]	50	166	46	3	Negative
$InsP_6$	328.9	481	50	166	10	1	Negative
$InsP_6$	328.9	78.9[a]	50	166	46	3	Negative
$[^{13}C_6]InsP_5$	292	504.7[a]	50	166	10	1	Negative
$[^{13}C_6]InsP_5$	292	78.9	50	166	14	1	Negative
$InsP_5$	288.9	498.7[a]	50	166	10	1	Negative
$InsP_5$	288.9	78.9	50	166	14	1	Negative
$InsP_4$	249	418.6[a]	50	166	10	1	Negative
$InsP_4$	249	320.6	50	166	14	1	Negative
$InsP_3$	419	320.6[a]	50	166	18	4	Negative
$InsP_3$	419	78.9	50	166	50	1	Negative

[a] MRM transition with the highest response for each compound (used for quantitation)

Table 1

Figure 1

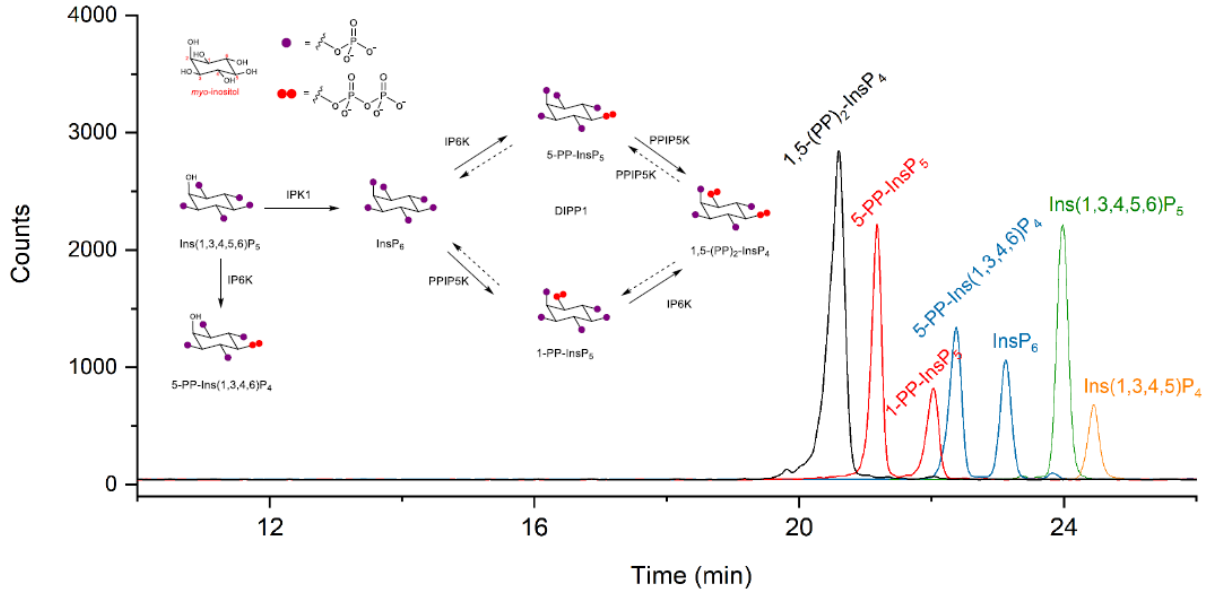

Figure 2

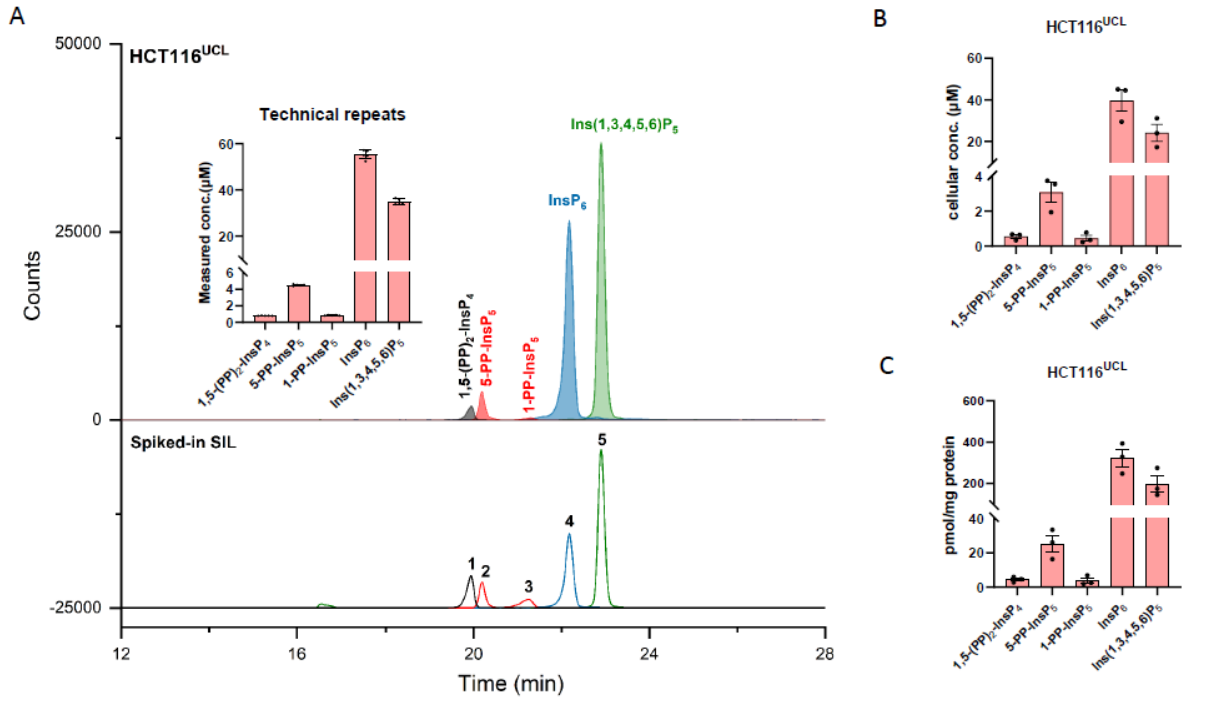

Figure 3

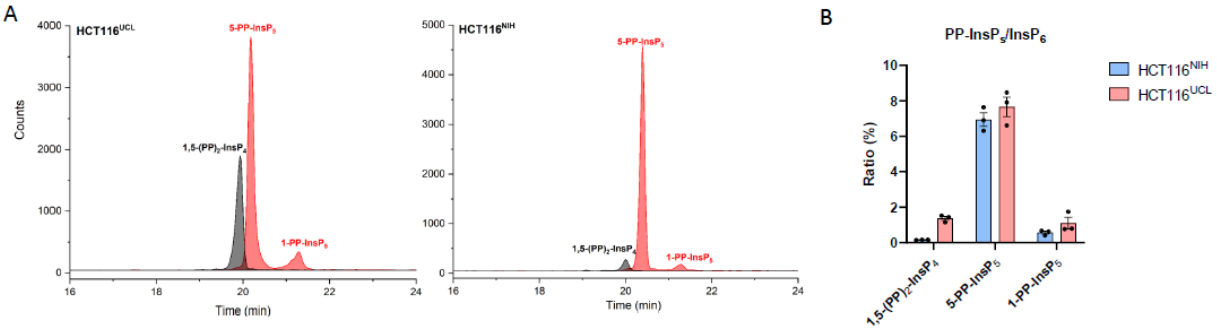

Figure 4