Review

Advances in phosphoinositide profiling by mass spectrometry

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Abstract Phosphoinositides (PIPs), which are phosphorylated forms of phosphatidylinositol (PI), are minor but essential components of membrane phospholipids. There are seven PIP classes differing in phosphorylation at the 3, 4, and 5 positions of the inositol ring of PI. Each PIP class plays crucial roles in various cellular events, such as signal transduction and membrane trafficking, while dysregulation of PIP metabolism is associated with the development of diseases such as cancer. Recent studies suggest the importance of the composition of the two fatty acyl chains in PIPs as well as the phosphorylation status of the inositol ring of PIPs. Therefore, there is an increasing demand for analysis of PIPs by mass spectrometry which can provide information on the fatty acyl chains of PIPs. Recently, mass spectrometric methods that can comprehensively analyze molecular species of all PIP regioisomers have been developed. In this short review, we describe past and present mass spectrometric analyses of PIPs as well as potential future improvements in the method.

Key words: phosphoinositides; regioisomer; methylation; supercritical fluid chromatography; chiral column chromatography

Introduction

Phosphoinositides (PIPs), which are phosphorylated forms of phosphatidylinositol (PI), are minor but essential components of membrane phospholipids in eukaryotes. Phosphorylation of PI at the 3-, 4-, and 5- hydroxyls of the inositol head group generates seven PIP classes, namely, PI(3)P, PI(4)P, PI(5)P, PI(3,4)P_2, PI(3,5)P_2, PI(4,5)P_2, and PI(3,4,5)P_3 (Fig. 1A). Each of the seven PIPs has a unique subcellular distribution with predominant localization in subsets of membranes: PI(4,5)P_2 localizes to the plasma membrane, PI(4)P to the Golgi apparatus and plasma membrane, PI(3,5)P_2 to late endosomes and lysosomes, and PI(3)P to early and late endosomes[1, 2] (Fig. 1B). PI(3)P is also produced in the endoplasmic reticulum during autophagy induction. PI(3,4)P_2 and PI(3,4,5)P_3 are produced at the plasma membrane in response to stimuli. Although the precise localization of PI(5)P is unclear, it has been found in diverse compartments, including the plasma membrane, early endosomes, and the nucleus[3]. These unique subcellular localizations are formed by the local actions of PIP kinases and phosphatases.

The unique subcellular localizations of PIPs allow them to act as signposts for each organelle, regulating the subcellular localization and function of various proteins involved in cell migration, adhesion, signal transduction, and membrane trafficking[2]. In addition, PI(4,5)P_2 is hydrolyzed by phospholipase C upon stimulation to produce inositol-1,4,5-triphosphate and diacylglycerol, both of which act as second messengers[4]. In the past decade, it has become clear that PI(4)P plays an essential role in intracellular lipid transport[2]. On the other hand, dysregulation of PIP metabolism is reportedly associated with the development of diseases[4]. For example, genes *PIK3CA*, *PTEN*, and *INPP4B*, which encode enzymes involved in the metabolism of PI(3,4,5)P_3, were found to be frequently mutated in many cancers[5].

PIPs are membrane lipids whose amounts are very small, making their measurement very difficult, and substantial

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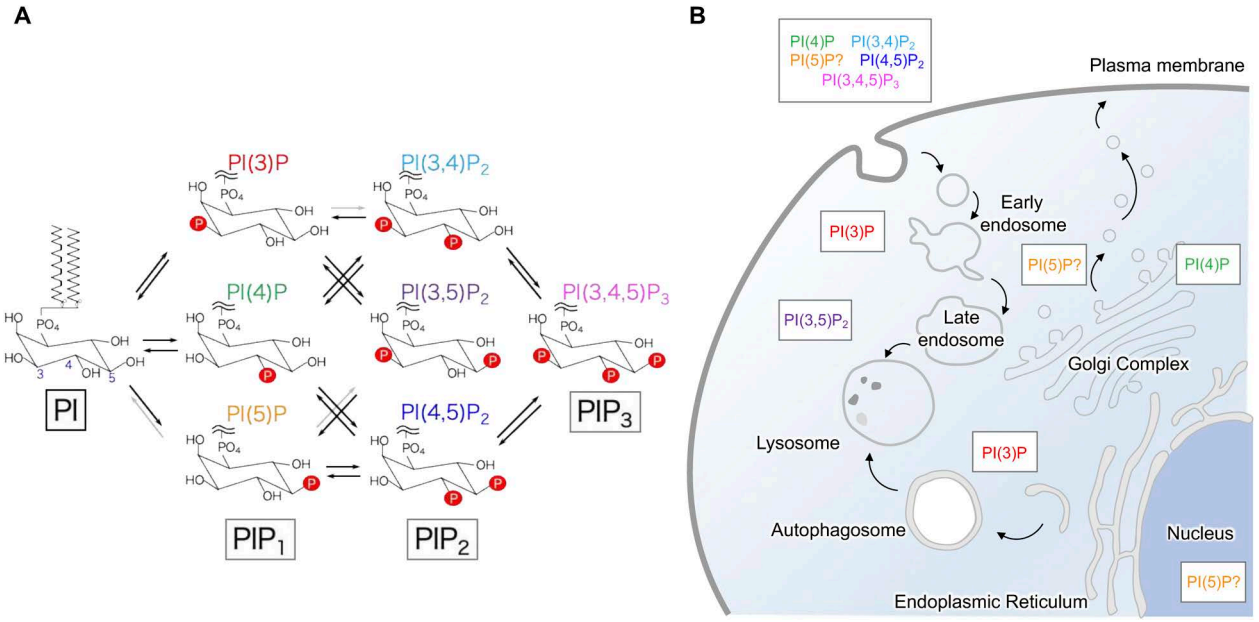

Fig. 1. Metabolism and subcellular localization of phosphoinositides.
A. Chemical structure of PI and PIPs. For PIPs, only the polar heads are shown. Addition and removal of phosphate groups at the 3′-hydroxy, 4′-hydroxy and 5′-hydroxy groups of PI by phosphoinositide kinases and phosphatases (solid arrows) creates seven distinct PIP species. B. Subcellular localization of PIPs. The spatiotemporally controlled activity of phosphoinositide kinases and phosphatases creates a distinctive enrichment of PIPs across the cellular compartments.

efforts have been made to measure them. Classically, cellular PIPs have been measured by labeling cells with [^{3}H]inositol or [^{32}P]phosphate and separating deacylated PIPs according to their polar head using high-performance liquid chromatography (HPLC)[6]. Non-radiolabeled PIPs can be measured by utilizing PIP kinase or PIP-binding proteins[7–9]. Although these methods can quantify the total amount of each PIP class, they do not provide information on fatty acyl chains.

Recently, it has become clear that the composition of fatty acyl chains of PIPs is associated with protein functions[10,11] and cancer progression[12], thus increasing the need for PIP profiling that includes information on fatty acyl chains. Electrospray ionization mass spectrometry (ESI-MS) is a powerful and commonly used method for quantifying individual lipid species[13,14]. In the past 20 years, several analytical techniques for PIPs by ESI-MS have been developed, enabling highly sensitive and versatile measurements of PIP molecular species. More recently, analytical methods that distinguish between positional isomers of PIPs have been developed. In this short review, we describe past and present mass spectrometric analyses of PIPs as well as potential future improvements in the method.

Profiling Intact PIPs by ESI-MS

Michelsen et al. applied ESI-MS for analyzing phosphatidylinositol-monophosphate (PIP_1) and phosphatidylinositol-bisphosphate (PIP_2) and measured 1-stearoyl-2-arachidonyl (18:0−20:4) species of PIP_1 and PIP_2 in bovine brain extracts[15]. They showed that about 200 pmol of PIP_1 and PIP_2 could be quantified by single ion monitoring in the negative ion mode. Hsu et al. intensively studied the detailed mechanism of fragmentation of phosphatidylinositol-4-phosphate [PI(4)P] and phosphatidylinositol-4,5-bisphosphate [$PI(4,5)P_2$] ions under collision-activated dissociation (CAD) with ESI-tandem MS (ESI-MS/MS), showing that ESI-MS/MS is a powerful tool for structural characterization of PIPs[16]. Profiling of PIP molecular species in biological samples by ESI-MS/MS was first reported by Wenk et al.[17]. They found that the addition of piperidine to the lipid samples markedly enhance the detection of PIP_1 and PIP_2 ions in the negative ion mode. They detected elevated levels of PIP_2 species in human fibroblasts from patients with Lowe syndrome, which is caused by mutations in *OCRL1*, which encodes a phosphoinositide 5-phosphatase[18]. They also showed that deficiency of enzymes involved in PIP metabolism in *Saccharomyces cerevisiae* affects not only the levels of PIPs but also PIP profiles, suggesting that PIP metabolizing enzymes are selective for cer-

Table 1. Advances in the analysis of PIPs by mass spectrometry

Authors	Year	Advances/Novelty	ref.
Michelsen et al.	1995	Application of ESI-MS to PIP_1 and PIP_2 analysis.	15
Hsu et al.	2000	The detailed mechanism of fragmentation of PI(4)P and $PI(4,5)P_2$ ions under CAD with ESI-MS/MS.	16
Wenk et al.	2003	Measurement of PIP_1 and PIP_2 acyl vriants in biological samples by ESI-MS/MS. Enhancement of PIPs signal intensities by piperidine.	17
Milne et al.	2005	Measurement of $PI(3,4,5)P_3$ molecular species in biological samples Development of 2-step extraction method.	19
Pettitt et al.	2006	Measurement of PIP molecular species and PIP_2 regioisomers by normal phase LC-MS. Development of buffered citrate extraction method. Use of ethylamine as a mobile phase modifier. Distinct fragmentation pattern of PI(4)P from other PIP_1s at MS^3.	20
Ogiso et al.	2008	Measurement of PIP molecular species by reverse phase LC-MS Use of DEAE column to obtain a PIP-rich fraction.	21
Clark et al.	2011	Development of PIP derivatization method with TMS-diazomethane. Measurement of methylated PIPs by LC-MS/MS.	22
Kim et al.	2017	Development of LC-MS/MS method for structural identification of *sn*-1 and *sn*-2 fatty acyl chains of PIPs. Enhancement of PIPs signal intensities by ammonium ion adduction.	23
Wang et al.	2016	Development of a method for quantifying each PIP2 regioisomer and PI(3)P by simulation using the information of methylation patterns.	24
Bui et al.	2018	Development of a technique for measuring non-derivatized PIP_2 regioisomers by RPLC-MS with the addition of the ion-pairing reagents.	25
Melek et al.	2017	Development of a method to distinguish between 18:0/20:4 $PI(4,5)P_2$ and $PI(3,4)P_2$ in biological samples by shortening the acyl groups of PIPs by ozone cleavage.	26
Li et al.	2021	Development of a method to separate all methylated PIP_1 and PIP_2 regioisomers by HPLC-MS using a cellulose-based chiral column Use of SWATH to obtain a comprehensive PIP profile.	27
Morioka et al.	2022	Development of a method for simultaneously quantifying molecular species of all seven PIP classes by HPLC-ESI-MS/MS using a cellulose-based chiral column.	28
Shimanaka et al.	2022	Development of a method for simultaneously quantifying molecular species of all seven PIP classes by SFC-ESI-MS/MS using a β-cyclodextrin column.	29

tain acyl chains or that organelles have a specificity of PIP profiles. Milne et al. reported profiling of $PI(3,4,5)P_3$ together with PIP_1 and PIP_2 in macrophages[19]. They used a selective two-step extraction, in which cells were treated with neutral solvents to extract unwanted neutral phospholipids, and then PIPs were extracted from the resulting pellet with acidic solvents. Their analysis revealed that different profiles of PIP_3 were produced in macrophages stimulated by different agonists, suggesting the activation mechanism of PI-3 kinase and/or $PI(4,5)P_2$ species that may serve as substrates are different for different agonists. Wenk et al. and Milne et al. used direct infusion mass spectrometry, in which ion suppression effects may affect the measurements. Pettitt et al. developed a normal phase LC-MS method to resolve PIP regioisomers[20]. They thoroughly investigated each step from the extraction to the final analysis of PIPs and made several improvements. Especially, they developed a highly-efficient buffered citrate extraction methodology to minimize acid-induced phosphoinositide degradation and a sensitive LC-MS method using ethylamine as a mobile phase modifier. They showed that levels of PIP_1 were 2.5 times higher than those of PI in human platelets. The detection limits were 250 fmol for PIP_1, 1 pmol for PIP_2, and 5 pmol for PIP_3. Reversed-phase (RP) LC is excellent for separating phospholipid molecular species and detecting minor components. Ogiso et al. developed a sensitive method to determine PIP_1, PIP_2, and PIP_3 profiles by RPLC-MS[21]. They used a (diethylamino)ethyl

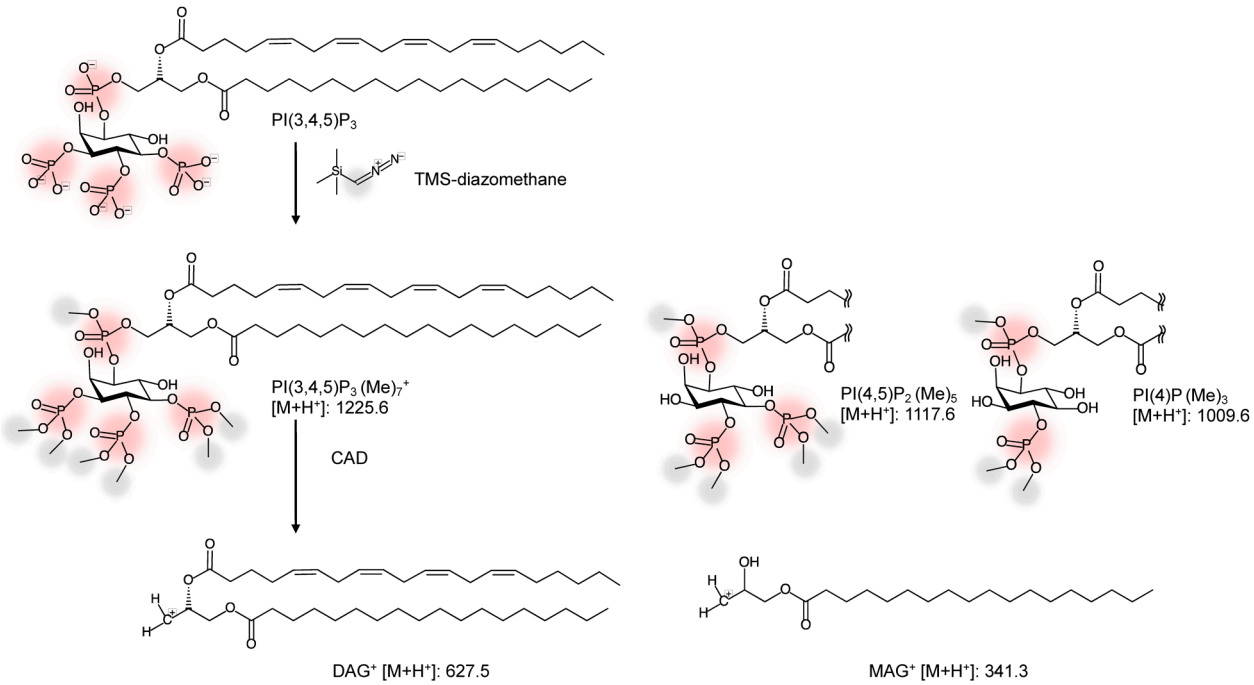

Fig. 2. Methylation of the phosphate groups of PIPs by TMS-diazomethane.
Treatment of PI(3,4,5)P_3 with TMS-diazomethane yields heptamethylated PI(3,4,5)P_3 [PI(3,4,5)P_3 $(Me)_7$]. In the case of PI(4)P and PI(4,5)P_2, trimethylated PI(4)P [PI(4)P $(Me)_3$]and pentamethylated PI(4,5)P_2 [PI(4,5)P_2 $(Me)_5$]are mainly produced, respectively. The methylated PIPs are readily detected as positive ions and collision-activated dissociation (CAD) of a methylated PIP ion yields diacylglycerol (DAG) ion and monoacylglycerol (MAG) ion.

(DEAE)-cellulose column to obtain a PIP-rich fraction from the lipid extracts and an alkaline-resistant C8 column. They measured 17 PIP_1 and PIP_2 species in epidermal growth factor (EGF)-stimulated A431 cells. They also showed that the level of 36:2 PIP_3 species increased upon EGF stimulation. The detection limit of their method was 25 fmol for each PIP. These and other advances in the mass spectrometry of PIPs (described below) are summarized in (Table 1).

Highly-sensitive PIP Analysis by Using a Derivatization Technique

As mentioned above, the analytical method for PIPs by ESI-MS, including sample preparation, has been greatly improved. However, there remain problems in PIP recovery and stability, which are associated with the inositol ring phosphate group being adsorbed on glass and metal surfaces and undergoing hydrolysis[20,21]. To address these problems, Clark et al. developed a derivatization method in which the phosphate group is methylated with trimethylsilyl-diazomethane[22] (Fig. 2). Derivatization of PIPs with TMS-diazomethane can be performed directly in the acidified chloroform-methanol phase from cellular or tissue extracts and takes only 10 min. The derivatization results in the addition of three methyl groups to PIP_1, five to PIP_2, and seven to PIP_3 (Fig. 2). Methylation of the phosphate groups improves the recovery of PIPs and renders the highly polar PIPs less charged and more stable. Methylated PIPs can be detected as positive ions by ESI-MS, and the CAD of methylated PIP ions leads to neutral loss of the methylated inositol phosphate headgroup, producing charged diacylglycerol fragments. Clark et al. used a C4 reversed-phase HPLC column to concentrate methylated PIPs and detected them by neutral loss scan mode or multiple reaction monitoring (MRM) mode using diacylglycerol ions as Q3 masses. Clark et al. quantified PI(3,4,5)P_3 species in fMLP-stimulated human neutrophils, glucose-stimulated human adipose tissues, and insulin-stimulated mouse liver. They also showed that 18:0/20:4 PI(3,4,5)P_3 accumulated selectively upon EGF stimulation in MCF10a breast cancer cells, although its precursor 18:0/20:4 PI(4,5)P_2 was the least abundant species of PI(4,5)P_2. Kim et al. developed an LC-MS/MS method for structural identification of *sn*-1 and *sn*-2 fatty acyl chains of PIPs[23]. They noted that *sn*-1 monoacylglycerol ions are generated by the CAD of methylated PIPs and performed precursor ion scans of monoacylglycerol ions as well as neutral loss scans of the

inositol head groups in positive ion mode. By comparing LC retention times between the two scan modes, they succeeded in determining *sn*-1 and *sn*-2 fatty acyl chains of PIP species. They also found that ammonium adduction enhanced signal intensities of PIPs by about twofold. Using their method, they quantitatively profiled PIP species in insulin-treated AML12 hepatocytes and mouse livers with structural information of the *sn*-1 and *sn*-2 fatty acid compositions.

Regioisomer-specific Analysis of PIP Molecular Species

As mentioned above, PIP_1 and PIP_2 each have three regioisomers, and each regioisomer is involved in distinct cellular functions. Therefore, each regioisomer must be quantified separately to investigate its functions. However, the masses of the PIP regioisomers are exactly the same, making it impossible to distinguish the regioisomers by mass spectrometry.

Pettit et al. reported a method for measuring non-derivatized PIP regioisomers by using normal-phase LC and ion trap mass spectrometry[20]. They showed that three PIP_2 regioisomers could be separated by normal phase LC using a silica column. Although the three PIP_1 regioisomers could not be separated by the LC system, PI(5)P eluted slightly ahead of PI(3)P and PI(4)P. Moreover, they found that the fragmentation pattern of PI(4)P at MS^3 was different from the fragmentation patterns of the other PIP_1 regioisomers, which allowed them to calculate the ratio of PI(4)P to other PIP_1 isomers present. Thus, the combination of the separation of PI(5)P by normal phase LC and the diagnosis of the presence ratio of PI(4)P and PI(3)P by ion trap MS enabled the measurement of each PIP_1 regioisomer. Using this method, they showed that PI(3,5)P_2 is increased in yeast cells upon hyperosmotic stress. Wang et al. found that PIP_2 regioisomers and PI(3)P exhibit unique methylation patterns and developed a method for quantifying each PIP_2 regioisomer and PI(3)P by simulation using the information of methylation patterns[24]. Since the methylation patterns of PI(4)P and PI(5)P were virtually identical, only their mixtures could be determined from biological samples by this method. They showed changes in the PIP profile in the brain cortices of *db/db* mice. Bui et al. reported a technique for measuring non-derivatized PIP_2 regioisomers by using C18 reverse phase chromatography with the addition of the ion-pairing reagents diisopropylethanolamine (DiiPEA) and ethylenediamine tetraacetic acid (EDTA)[25]. Melek et al. developed a method to distinguish between 18:0/20:4 PI(4,5)P_2 and PI(3,4)P_2 in biological samples by shortening the acyl groups of PIPs by ozone cleavage[26]. Thus, while various efforts have been made to measure each PIP regioisomer separately, there is much interest in the development of a simple and versatile method to separate all PIP regioisomers by chromatography and measure them simultaneously.

Very recently, three independent groups, including ours, have reported a method for the simultaneous determination of all PIP regioisomers by chromatography-tandem mass spectrometry[27–29]. Li et al. successfully separated all methylated PIP_1 and PIP_2 regioisomers by HPLC using a cellulose-based chiral column[27]. They also utilized SWATH (sequential window acquisition of all theoretical mass spectra) technology, a data-independent acquisition technique, to obtain a comprehensive PIP profile. Using their method, they reported the full PIP profiles of human plasma, *Pichia pastoris*, and HeLa cells, including profile changes upon treatment with PI3K inhibitor wortmannin. Morioka et al. also reported a method for simultaneously quantifying molecular species of all seven PIP classes by HPLC-ESI-MS/MS using a cellulose-based chiral column[28]. Their method revealed (1) a severe skewing in acyl chains in PI(3,4)P_2 and PI(3,4,5)P_3 in *Pten*-deficient prostate cancer tissues, (2) extracellular mobilization of PIPs upon expression of oncogenic *PIK3CA*, and (3) a unique profile for exosomal PIPs. Although both two methods use cellulose-based chiral columns, the order of elution of the PIP_1 regioisomers is different: PIP_1 regioisomers elute in the order PI(3)P, PI(5)P, and PI(4)P in the method of Li et al., but in the order PI(3)P, PI(4)P, and PI(5)P in the method of Morioka et al. PIP_2 regioisomers elute in the order PI(3,5)P_2, PI(3,4)P_2, and PI(4,5)P_2 for both methods.

As an alternative chromatographic approach, we developed a method for separating methylated PIP regioisomers by supercritical fluid chromatography (SFC)[29]. A supercritical fluid is a fluid that is maintained at a temperature and pressure above its critical point. It has viscosity and diffusivity close to gas and has solubility close to liquid. Because of these characteristics, SFC enables fast and efficient separation. In addition, SFC and HPLC have different separation behaviors, allowing SFC to separate compounds that cannot be separated by HPLC. We found that SFC with a β-cyclodextrin column successfully separated methylated

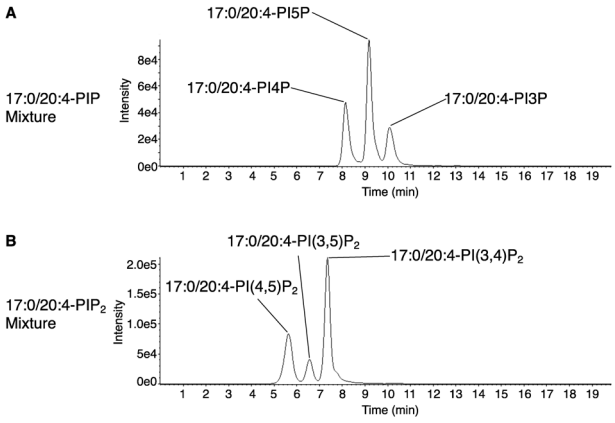

Fig. 3. Separation of PIP_1 and PIP_2 regioisomers by SFC. Synthetic 1-heptadecanoyl-2-arachidonyl (17:0/20:4) PIP standards were methylated with TMS-diazomethane and analyzed by SFC-ESI-MS/MS. MRM chromatograms of 17:0/20:4-PIP_1 (A) and 17:0/20:4-PIP_2 (B) are shown.

PIP_1 and PIP_2 regioisomers (Fig. 3). The SFC-based method produces a different elution profile for the PIP regioisomers than the two HPLC-based methods: in the SFC-based method, the PIP_1 regioisomers elute in the order PI(4)P, PI(5)P, PI(3)P, and the PIP_2 regioisomers elute in the order PI(4,5)P_2, PI(3,5)P_2, PI(3,4)P_2. In the HPLC-based method, the PIP_1 and PIP_2 regioisomers elute in a narrow range of retention times, whereas, in the SFC-based method, the PIP_1 and PIP_2 regioisomers elute in a broader range, resulting in a better separation of regioisomers. The SFC system coupled with ESI-MS/MS makes it possible to measure the molecular species of all seven PIP classes in the biological samples. Profiling PIP molecular species in mouse tissues by this method revealed that PIPs with two saturated fatty acids, which were hardly detected in most of the tissues or cultured cells, were abundant in the testis. We also found that less unsaturated PI(3)Ps such as 34:1, 34:2, 36:1, and 36:2 PI(3)P, which are probably derived from newly synthesized PI, selectively increased during autophagy induction. Thus, chiral column chromatography has made it possible to measure the molecular species of all seven PIP classes in a single analysis.

Future Perspectives

PIP analysis has now reached a major milestone with methods that can measure the molecular species of all seven PIP classes. Analyzing full PIP profiles in biological samples should lead to a better understanding of the physiological and pathological roles of PIPs at the molecular species level. However, there remains room for improvement in the absolute quantification of PIP molecular species in biological samples. Because PI(4)P and PI(4,5)P_2 are far more abundant than other PIPs in biological samples, minor PIP_1s [PI(5)P and PI(3)P] and minor PIP_2s [PI(3,4)P_2, and PI(3,5)P_2] ended up on the shoulders of the large PI(4)P and PI(4,5)P_2 peaks, respectively, preventing their accurate quantification of minor PIPs. In fact, the amounts of minor PIPs [PI(5)P, PI(3)P, PI(3,4)P_2, and PI(3,5)P_2] in the biological samples measured by HPLC-ESI-MS/MS or SFC-ESI-MS/MS were much higher than those measured by radioisotope-labeling methods[27,29]. Although this may be partly due to the effect of culture conditions during radioisotope labeling on PIP metabolism, we cannot rule out the possibility that peaks of minor PIP_1 and PIP_2 classes are inflated by those of PI(4)P and PI(4,5)P_2. Further improvements in separating PIP regioisomers would allow the absolute quantification of PIP molecular species in biological samples.

Recent advances in phospholipid mass imaging techniques have made it possible to image various phospholipid molecular species in tissues at the single-cell level[30,31]. However, there are few reports on MS imaging of PIPs[32,33] and none on MS imaging of PIP regioisomers. For MS imaging of PIP regioisomers, it is necessary to separate PIP regioisomers inside the mass spectrometer. One powerful approach for isomer-specific imaging of PIPs would be ion mobility spectrometry, which is a gas-phase separation technique in which ion mobility is affected by the molecular shapes of ions[34]. Ion mobility spectrometry will also help the absolute quantification of PIP regioisomers by LC/SFC-ESI-MS/MS. By incorporating new methodologies such as ion mobility spectrometry, the analysis of PIPs by MS will overcome the current challenges and further contribute to our understanding of the physiological and pathological roles of PIPs.

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Conflict of Interest

NK is a co-inventor of a Japan patent 7017704 with the title "Biological membrane phosphoinositide separation method". All other authors declare no competing interests.

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