**STANDARDIZED MEASURE FOR
PHOSPHATIDYLINOSITOL 4,5-BISPHOSPHATE BY MASS SPECTROMETRY**

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Abstract

STANDARDIZED MEASURE FOR PHOSPHATIDYLINOSITOL 4,5-BISPHOSPHATE BY MASS SPECTROMETRY

Jaspreet Jawanda, Ryerson University, 2013, Masters of Science in Molecular Science

Phosphoinositides (PIs) play a fundamental role in many physiological processes such as cell surface signal transduction, membrane trafficking, cytoskeleton regulation, nuclear events and permeability and transport functions of membranes. Levels of PIs vary under different physiological conditions thus PI profiling may be an important step in elucidation of importance in the progression towards many diseases. Previous methods for PI analysis have several disadvantages in time-constraints, use of radioactive samples and inconsistent results due to lack of sensitivity. Mass spectrometry has previously been utilized to quantify low abundance peptides, metabolites and lipids. Here we propose a novel approach for PI quantitation based on inositol head cleavage coupled to high-performance liquid-mass spectrometry (HPLC-MS) to overcome these issues. First, we highlight the extraction and deacylation of PIs from *S. Cerevisiae*, followed by purification via reverse phase chromatography with a Sep-Pak. Next, using commercially purchased PI(4,5)P₂ standards, we created a tune file which provide the correct conditions sensitive enough to identify prolific ion peaks to be utilized in characterization of biological samples. Using the standard tune file, we successfully identified and quantitated the PI(4,5)P₂ in cells lacking INP51 which have 2-4 fold increase in PI(4,5)P₂ and comparing them to the wild type cells. The methods described may form a basis for further optimization of mass spectral based quantitation of PIs.

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Abbreviations

5-p	Inositol polyphosphate 5-phosphatase
Ins	Inositol
CID	Collision induced dissociation
EDTA	Ethylenediaminetetraacetic acid
DAG	Diacylglycerol
GFP	Green fluorescent protein
Ins(1,4,5)P ₃	Inositol-1-4-5-trisphosphate
Ins	Inositol
m/z	Mass/charge
DMHA	Dimethylhydroxylamine
EI	Electron Ionization
DNA	Deoxyribonucleic acid
ESI	Electrospray Ionization
MS	Mass spectrometry
FAB	Fast atom bombardment
MALDI	Matrix-assisted laser desorption ionization
AcN	Acetonitrile
DC	Direct current
HPLC	High pressure liquid chromatography
LLE	Liquid-liquid extraction
SPE	Solid-phase extraction
SIM	Selected ion monitoring
SRM	Selected reaction monitoring
LMPD	Lipid maps proteome database
PI	Phosphoinositides
PtdIns3P	Phosphatidylinositol-3-phosphate
PtdIns(3,5)P ₂	Phosphatidylinositol-3-5-bisphosphate
PtdIns(4,5)P ₂	Phosphatidylinositol-4-5-bisphosphate
PtdIns(3,4,5)P ₃	Phosphatidylinositol-3-4-5-trisphosphate
PtdIns4P	Phosphatidylinositol-4-phosphate
PtdIns5P	Phosphatidylinositol-5-phosphate
PtdIns(3,4)P ₂	Phosphatidylinositol-3-4-bisphosphate
RF	Radio frequency
MeOH	Methanol
ddH ₂ O	Double distilled Water
FA	Formic acid
RNA	Ribonucleic acid
TLC	Thin layer chromatography
YP	Yeast-peptone
YNB	Yeast nitrogen base
SD	Selective drop-out
PCA	Perchloric acid
SD	Selective drop-out
M1	Mobile phase A: H ₂ O + 0.1% ammonium hydroxide

M2

Mobile phase B: 50% isopropanol +50% methanol + 0.1% ammonium hydroxide

Mobile phase A: water + 5mM N,N-dimethylhexylamine + 4mM glacial acetic acid

Mobile phase B: methanol + 5mM N,N-dimethylhexylamine + 4mM glacial acetic acid

Chapter 1: Introduction

Advances in separation technology and particularly in mass spectrometry have revolutionized the analysis and quantification of cellular and tissue lipids. Due to the collective effort of the scientific community, this has led to continual growth in expanding the field of studying lipids thus, giving birth to many databases such as lipid maps proteome database (LMPD), lipid library, and lipid bank (Table 1.1).

Resource	URL	Country	Comments
Lipid Library	http://lipidlibrary.aocs.org/	United Kingdom	Multiple reference topics and examples, mass spectrometry (MS) and nuclear magnetic resonance (NMR) libraries, literature service
CyberLipid Center	http://www.cyberlipid.org/	France	Description of lipid classes with examples and literature references
LipidBank	http://lipidbank.jp	Japan	Lipid database with a wide variety of categories, including plant lipids
LIPIDAT	http://www.lipidat.chemistry.ohio-state.edu	United States	Database composed mostly of phospholipids and associated thermodynamic data
Lipid Maps	http://www.lipidmaps.org/	United States	Classification scheme, lipid and protein databases, MS libraries, search tools

Table 1.1: Online resources for Lipid Databases. Online resources available for comprehensive classification schemes for lipids are summarized.

Lipidome

Just as the genome is the complete gene profile within an organism, the lipidome is the complete lipid profile within a cell. Lipids are a major constituent of a cell and serve an important function as energy reservoirs, vital secondary messengers (generated by a variety of intracellular enzymes) and formulation of a cellular membrane (Wenk, 2005). Lipids are tightly regulated molecules within cells thus collapse of the regulatory measures can have profound changes within cells. The effects of this include detrimental diseases such as diabetes, obesity, cancer and many neurodegenerative disorders including schizophrenia, Alzheimer's disease, Parkinson's disease, and Niemann-Pick disease (Cutler *et al.*, 2004; Pentchev *et al.*, 1985; Sturley *et al.*, 2004; Wenk, 2005).

As lipids serve multiple unique functions within cells, logically, numerous types exist. Based on their hydrophobicity/hydrophilicity profiles, lipids can be classified into eight different categories: fatty acids, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids, and polyketides (Fahy *et al.*, 2005). These lipids vary in molecular composition and relate by their solubility in nonpolar organic solvents such as ether, chloroform, acetone and benzene and generally their insolubility in water. The inositol containing glycerophospholipids known as phosphoinositides (PI), which can be deacylated to form glycerated phosphoinositols will be the main focus of this project.

Phospholipids

Underneath the umbrella of the lipidome, exists a major class of lipids known as phospholipids. Phospholipids are amphipathic molecules meaning they contain both hydrophobic and hydrophilic ends. Due to hydrophobic interactions, phospholipids arrange into a lipid bilayer where the hydrophobic ends are oriented towards the middle of

the layer (Fig 1.1). This is important as it provides a thin barrier around cells keeping ions, proteins and any water-soluble molecules from diffusing through the hydrophobic portion of the lipid bilayer.

Phospholipids make up the largest portion (60%) of the lipid mass of the eukaryotic cell membrane (Han *et al.*, 2005). Hence, alterations in phospholipid composition may lead to profound changes within the cell. Phospholipids are important constituents in cellular membrane formation, cellular signaling and trafficking, intercellular adhesion and cytoskeleton support, in addition to acting as cellular mediators (Pulfer *et al.*, 2003). Currently, there are over 100 individual molecular species of phospholipids that can be distinguished using analytical techniques (Peterson and Cummings, 2006).

Phospholipids are categorized into multiple classes based on their chemical structures with a nomenclature system based on the nature of their polar head groups at the sn-3 position (Fig 1.3) (Fahy *et al.*, 2005; Han *et al.*, 2005). There are five general classes of phospholipids: phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine, phosphatidylsphingomyelin and phosphatidylinositol (PtdIns). The special characteristics of the different classes of phospholipids depend upon the element attached at the head i.e., serine, choline, ethanolamine or inositol. PIs have an attached myo-inositol headgroup. The names and head groups attached are summarized in Fig 1.2.

Fig 1.1: Structure of a Phospholipid. A phospholipid consists of a polar head group, connected by a glycerol to two fatty acid tails. The polar head group is hydrophilic while the fatty acid chains are hydrophobic, repelling water. A lipid bilayer is formed in water when the hydrophobic tails line up against one another, forming a membrane of hydrophilic heads on both sides facing the water (Adapted from: Nelson, 2008).

name of X-OH	formula of X	name of phospholipid
water	—H	phosphatidic acid
ethanolamine	—CH ₂ CH ₂ NH ₃ ⁺	phosphatidylethanolamine
choline	—CH ₂ CH ₂ N(CH ₃) ₃ ⁺	phosphatidylcholine
myoinositol		phosphatidylinositol
glycerol	—CH ₂ CH(OH)CH ₂ OH	phosphatidylglycerol

Fig 1.2: Characterization of phospholipids. Phospholipids are characterized by the structural element attached to the head group of the general phospholipid structure which consists of four components: fatty acids, a platform to which fatty acids are attached; glycerol backbone, a phosphate and an alcohol attached to the phosphate (Adapted from Berg, 2010).

Phosphatidylinositol (PtdIns) and Phosphoinositides (PIs)

The role of membrane PIs has extended well beyond intracellular signaling pathways and now includes the modulation of cytoskeleton organization, apoptosis, and membrane trafficking (Payrastre, 2001; Corvera, 1999; Cremona, 2001). These molecules are also known to play a significant role in the nucleus (Corvera, 1999).

Family of the PIs contains the non-phosphorylated precursor PtdIns and seven phosphorylated derivatives with different phosphorylation patterns on the myo-inositol ring phosphorylated by specific kinases: phosphatidylinositol-3-phosphate (PtdIns3P), phosphatidylinositol-4-phosphate (PtdIns4P), phosphatidylinositol-5-phosphate (PtdIns5P), phosphatidylinositol-3-4-bisphosphate (PtdIns(3,4)P₂), phosphatidylinositol-3-5-bisphosphate (PtdIns(3,5)P₂), phosphatidylinositol-4-5-bisphosphate (PtdIns(4,5)P₂), and phosphatidylinositol-3-4-5-trisphosphate (PtdIns(3,4,5)P₃) (Fig 1.3).

PI's are responsive to cellular signals and metabolic interconversions can generate any of the other family members via various kinase and phosphatase enzyme processes (Fig 1.4). Specifically in mammals, there are 47 genes encoding 19 phosphoinositide kinases and 28 phosphoinositide phosphatases, responsible for the 18 different interconversion reactions known to date (Sasaki *et al.*, 2009). Understanding the distinct and overlapping functions of these phosphoinositides is important to gain insight into both normal function and human diseases.

Fig 1.3: A) Precursor and B) derivatives of phosphoinositides. A) Structurally, PIs contain a polar inositol head group with at least one phosphate group on the sn-1 and sn-2 position of a glycerol backbone linked to a fatty acid chain. The myo-inositol head group contains five free hydroxyl groups but only three of them (D-3, D-4, and D-5) can have sequential and reversible phosphorylation by specific kinases. B) Schematic representation of the seven derivatives of phosphoinositides. Phosphoinositol can be phosphorylated to form seven derivatives (Adapted from Sasaki *et al.*, 2009).

Fig 1.4: Conversion of phosphoinositides. Representation of the metabolic interconversions that generate seven phosphoinositides. These can be interconverted between each other with the help of different kinases and phosphatases. The dashed reactions are yet to be documented (Adapted from Sasaki *et al.*, 2009).

Although PI's are known to play a fundamental role in many cellular physiological processes such as signal transduction at the cell surface, acquisition of cellular polarity, cytoskeletal dynamics, regulation of cellular adhesion, motility and cytokinesis, they represent less than 1% of lipids in resting mammalian cells (Di Paolo *et al.*, 2006; Krauss *et al.*, 2007; Odorizzi *et al.*, 2000; Martin, 1998) (Fig 1.5).

Fig 1.5: Cellular location of phosphoinositides within the cell. The schematic figure shows the subcellular distribution of phosphoinositide species. PtdIns(4,5)₂ is primarily localized and concentrated in cytosolic membranes, possibly in raft-like domains. PtdIns(4,5)₂ is also a key regulator in aspects of gene expression (Kutateladze, 2010).

Phosphoinositide signaling occurs through binding of specific domains found on cytosolic proteins or cytosolic domains of membrane proteins (Lemmon, 2003; Balla, 2005). Proteins interact with PI's in clusters of basic residues within unstructured regions (profilin) or folded modules (PH domain) (Hurley, 2001). Most PIs have distinct conserved PI-binding domains, which may have overlapping specificities such as the PH, GRAM, ENTH, PHD, and PX (Rusten, 2006; Lemmon, 2003; Downes *et al.*, 2005; Balla, 2005; Stenmark *et al.*, 2002) (Table 1.2). The presence of these domains allows the recruitment of host proteins to specific regions within the cell via interactions with PIs and act as allosteric activators or inhibitors for protein-protein interactions and enzyme activity (Cullen *et al.*, 2001; Hurley and Meyer, 2001).

Module	Specificity	Examples of Proteins
A/ENTH	PtdIns(4)P	EpsinR
	PtdIns(3,5)P ₂	Ent3p,, Ent5p
	PtdIns(4,5)P ₂	AP180, CALM, epsin, HIP
C2	PtdIns(4,5)P ₂	Synaptotagmin
FERM	PtdIns(4,5)P ₂	Ezrin, moesin, radixin, talin
FYVE	PtdIns(3)P	EEA1, Hrs, SARA, PIKfyve
GRAM	PtdIns(3,5)P ₂	Mytubularin
PDZ	PtdIns(4,5)P ₂	Syntenin
PH	PtdIns(4)P	FAPP1/2, OSBP
	PtdIns(3,4)P ₂	AKT/PKB, TAPP1,2
	PtdIns(4,5)P ₂	PLCδ1, dynamin
	PtdIns(3,4,5)P ₃	BTK, AKT/PKB, ARNO, GRP1
PHD	PtdIns(5)P	ING2
PTB	PtdIns(4,5)P ₂	Dab1, ARH, SHC
	PtdIns(3,4,5)P ₃	SHC
PX	PtdIns(3)P	SNX2,3,7,13
	PtdIns(5)P	SNX13
	PtdIns(3,4)P ₂	p47PHOX
	PtdIns(4,5)P ₂	Class II PI(3)kinase
	PtdIns(3,4,5)P ₃	CISK

Table 1.2: Phosphoinositide Domains. Phosphoinositide binding modules and their reported phosphoinositide binding preferences (Adapted from Di Paolo and De Camilli, 2006).

PtdIns(4,5)P₂

Of the seven PI species, this study focused on PtdIns(4,5)P₂ (Fig 1.6). PtdIns(4,5)P₂, has been shown to be localized within the plasma membrane and nucleus in high concentrations (Aikawa and Martin, 2003). Additionally, it's involved in endocytosis and cytoskeletal dynamics by binding to proteins with PH and ENTH domains (Raucher *et al.*, 2000).

Fig 1.6: Structure and roles of PtdIns(4,5)P₂. PtdIns(4,5)P₂ is important in many cellular processes such as cell survival, gating of ion channels and transporter, vesicle trafficking and actin cytoskeleton remodeling (Adapted from Krauss and Haucke, 2007).

PtdIns(4,5)P₂ is predominantly synthesized by type I phosphatidylinositol 4-phosphate 5-kinases using PtdIns(4)P as a precursor (Fig 1.7). As a signaling molecule, via the catalytic action of phosphoinositide phospholipase C (PLC), PtdIns(4,5)P₂ is hydrolyzed into two secondary messengers: inositol-1,4,5-trisphosphate (Ins(1,4,5)P₃) and diacylglycerol (DAG) (Delage *et al.*, 2013). Additionally, PI(4,5)P₂ can be further phosphorylated by class I PI 3-kinase to form PtdIns(3,4,5)P₃ that activates downstream signaling components important in cell survival and growth (Delage *et al.*, 2013). Both PI(4)P and PI(5)P are precursors for PI(4,5)P₂.

PtdIns(4,5)P₂ mediated signaling events are initiated by specific PI kinases and are terminated in part by a family of PI 5-phosphatases (5-Pases). PI 5-Pases regulate cellular levels of PtdIns(4,5)P₂ by removing the phosphate at the D5 position of the inositol head group (Majerus *et al.*, 1999). Several mammalian 5-Pases have been identified thus far with various substrate specificities for PtdIns(4,5)P₂, PtdIns(3,4,5)P₃, and soluble inositol

phosphates (Stefan et al., 2002). Four PI 5-Pases are present in the yeast *Saccharomyces cerevisiae*. Three enzymes, Sjl1p, Sjl2p, and Sjl3p (also named Inp51p, Inp52p, and Inp53p), contain a Sac1-like domain and a 5-Pase domain (Stefan et al., 2002). Consistent with these in vitro activities, deletion of SJL1 leads to an increase in cellular PtdIns(4,5)P₂ levels whereas double deletion of SJL2 and SJL3 causes an increase in PtdIns(3,5)P₂ levels in vivo (Stefan et al., 2002).

Fig 1.7: A) Structure and B) the metabolic pathway of the production of PtdIns(4,5)P₂. The metabolic pathway is represented showing the interconversion of the different phosphoinositides. Lipid kinases phosphorylate the inositol ring at specific positions to form more highly phosphorylated forms such as PtdIns(4,5)P₂, while the lipid phosphatases dephosphorylate the inositol ring. Question marks indicate speculative pathways for which no enzyme has been identified (Skwarek and Boulianne, 2009).

PI Quantitation

Alterations in PI's levels can significantly impact cellular behaviour. In humans, inappropriate changes may manifest as diseases such as Alzheimer's, atherosclerosis, diabetes, obesity, and even cancer (Skwarek and Boulianne, 2009). Thus, the ability to quantitate and quantify the levels of PI's within biological samples may lead to progression in identifying important biomarkers for such diseases. Investigating lipid species that are localized within distinct parts of the cell can provide insight into areas such as antibody production, protein/enzyme interactions, drug/inhibitor interactions, specific metabolite pathways and finally differentiating between healthy vs. disease state cells (Harkewicz and Dennis, 2011; Rusten and Stenmark, 2006).

Despite technological advancements, the quantitation of PIs has been at a stalemate. Previously, PIs analysis has involved a) metabolic labeling with ^3H -inositol or organic ^{32}P followed by head group hydrolysis and chromatographic separation of PIs, b) transfection with fluorescent proteins such as Green Fluorescent protein (GFP) followed by fluorescence for effector proteins of PIs, and c) direct labeling of fixed cells with recombinant PIs binding domains or antibodies directed against specific PIs (Rusten and Stenmark, 2006; Kiefer *et al.*, 2010; Wakelam and Clark, 2011). These techniques are thought to be disadvantageous, as they present time-constraints, utilize radioactive samples and provide overall inconsistent results due to lack of sensitivity thus, not providing the exact relative content of PIs in a cellular extract. Although there is clear evidence of the importance of PIs, there has been limited improvement and progress in determining the cellular amounts and distinguishing individual species (structural differences) of these complex phospholipids. A fundamental reason for this is the low

concentration of PI's relative to other phospholipids in the cell coupled with loss occurring during extraction due to binding of the highly charged head groups to surfaces frequently used in lipid extraction and separation (protein bound during extraction process).

With the progress in studying lipids, the application of mass spectroscopy may resolve some of the issues highlighted above. Mass spectrometry has previously been utilized to quantify low abundance peptides, metabolites and lipids (Wenk, 2005). Previously, it has also been able to distinguish between the various phospholipids in biological samples. This may enable complete profiling of all phospholipids, both major and minor, from a single sample within a single analytical run (Kiefer *et al.*, 2010).

Mass Spectrometry of PI's

Mass spectrometry (MS) is a powerful analytical tool that can be used for the quantitative (concentration) and qualitative (structural) analysis of phospholipids including glycerophospholipids and sphingomyelin (Ho *et al.*, 2003). Here, molecules of interest are first introduced into the ionization source where they pass through a high voltage of approximately 5 kV forming either a negative or positive charge. Depending on the target mass to charge ratio (mass of the ion on the atomic scale divided by the number of charges that the ion carries; m/z), the ions arrive at different parts of the detector, finally the computer system assembles a mass spectrum showing the relative abundance of the signals. A MS typically consists of four components: an inlet for sample injection, the ion source, the mass analyzer and an ion detector (Fig 1.8). However, samples injected into the MS need to be entirely pure and clean in order to eliminate false positives or background noise that might otherwise alter the results. Hence, there are various separation and purification steps taken prior to injection into the source head (Fig 1.9).

Fig 1.8: Components of an electrospray ionization mass spectrometer (ESI-MS). The first major part is the inlet for sample injection. Ion source produces gaseous ions from the substances being studied. Analyzer resolves the ions into their characteristic mass components according to their mass-to-charge ratio. Detector system detects the ions and records the resolved ionic species. Finally, the computer produces a chromatogram of the results (Adapted from Harkewicz, 2011).

Fig 1.9: Extraction and recovery of phosphoinositides. The above procedure provides a very high extraction recovery from a variety of biological matrices and enable chromatographic resolution of most deacylated phosphoinositides.

Lipid Sample Preparation Techniques

Analysis of PIs through MS has been very difficult due to the large variety of PIs present. This is due to the complex nature of these molecules that are also closely similar in composition and mass isomers. There have been two fundamental approaches in quantitation and differentiation of these isomers by MS. The first method employs direct infusion (also known as the shot gun approach) of a biological sample into the MS (Milne *et al.*, 2005; Pulfer and Murphy, 2003; Wenk *et al.*, 2003; Postle *et al.*, 2007; Ejsing *et al.*, 2006). This results in comprehensive profiling of all intact PIs in the test sample. Although less time-consuming, this approach results in extremely complex spectra due to the plethora of closely related lipids that only vary by their lipid moieties. Consequently, till date, only lipids in platelets have been identified using this approach since platelets exhibit a far lower level of complexity for their PI's (Pettitt, 2006; Wakelam and Clark, 2011). An alternative to direct infusion involves the analysis of the head group following the cleavage from the lipid moiety and separation of the inositol head groups by HPLC (Kiefer *et al.*, 2010) (Fig 1.10).

Deacylation of Lipids

With deacylation, much of the complexity in analysis using the shotgun approach can be eliminated thus providing a more suitable approach if the focus lies on the changes of phosphorylation pattern of the head group within cells. The head group analysis method involves the deacylation of the lipids by cleaving the lipid chains producing glycerated inositol head groups (Fig 1.10). The reaction is an O-N-transacylation with N-methyl fatty acid amides being formed using methylamine (Clarke and Dawson, 1981).

Fig 1.10: Deacylation of phosphoinositides using methylamine (alkaline O-N-transacylation). The above representation shows a structure of a phosphoinositide and the deacylation steps, which results in the cleavage of the lipid moiety. Methylamine acts as a nucleophile breaking the acyl bond and releasing the fatty acid chain. The reaction is primarily an O-N-transacylation with N-methyl fatty acid amides being formed with an alcohol.

Lipid Extraction

Liquid-liquid extraction (LLE)

Extraction of PI's for analysis requires cell lysis and separation from other biomolecules that might interfere with the chromatography steps. Extraction of PIs has been performed by two different methods: a) solid-phase extraction (SPE) or b) liquid-liquid extraction (LLE). Given the diversity of phospholipids, there has to be a number of extraction steps in order to purify the lipids of interest.

Quantitative isolation of lipids from non-lipid contaminants must be accomplished before analysis is attempted. This is where the use of appropriate solvent systems is vital for the extraction of lipids from a biological milieu. LLE consists of placing the biological extracts into two immiscible liquids, which will ultimately form two separate phases. The

Bligh and Dyer method is one of the most common extraction procedure used for lipids as it provides optimum yield of representative lipids (Fig 1.11). This method consists of a solvent system of methanol, water and chloroform. This produces a biphasic system containing the chloroform layer that contains the lipids and the MeOH and water layer containing the additional cellular extract (Folch, 1957; Bligh, 1959).

Fig 1.11: Bligh and Dyer Method. Lipids are extracted by the Bligh and Dyer method. The biological cell extract is diluted with water and chloroform to achieve a biphasic system (chloroform/methanol/water). The upper methanol-water layer contains the non-lipids such as salts and sugars whereas the chloroform contains the lipids. Cell debris and protein are pelleted at the bottom of the tube.

Deacylated lipids producing highly polar head groups can be extracted using a similar LLE method. In this system, the inositol head groups are extracted through the aqueous layer.

Solid Phase Extraction (SPE)

An alternative separation method of phospholipids, SPE consists of a solid phase and a liquid phase which allows the separation of phospholipids based on their chemical properties (Fig 1.12).

Fig 1.12: General elution protocol for reverse phase chromatography on Sep-Pak cartridges C₁₈. The bonded phase is solvated and then the sample is loaded. The cartridge is washed with water to elute the unwanted components. Progressively stronger solvents can be used to elute components of interest (Waters Sep Pak manual).

The solid phase utilizes a cartridge consisting of an adsorbent support material like silica whereas the liquid phase consists of various concentrations of organic solvents that are used to elute out the phospholipids from the column, based on their chemical or structural properties (methanol, chloroform or hexane). This method is usually best used for further purification of small sample sizes as too large of a sample can saturate the column entirely, hence reducing the recovery of the phospholipid of interest. Once the lipids are extracted they can be further separated to obtain the phospholipid of choice.

Thin Layer Chromatography (TLC)

The term thin layer chromatography is used to describe a technique in which mixtures of different compounds are separated into individual substances using a stationary phase consisting of C₁₈ silica bound to an aluminum plate and a mobile phase that is drawn by capillary action. In the field of lipids, TLC is classically used for routine separations,

identification of the individual lipids and their quantitative determinations (Fuchs et al., 2011).

Lipid Separation

Lipids can be separated based on their physical and chemical properties using chromatography, a method commonly used to separate mixtures based on various factors such as polarity and molecular mass (similar principle used in SPE). Chromatography consists of a stationary and a mobile phase. A variety of chromatographic methods such as gas chromatography (GC), thin layer chromatography (TLC)) and high-pressure liquid chromatography (HPLC) can be used for the separation of phospholipids. However, HPLC using reverse phase chromatography is considered the most efficient chromatography method to separate deacylated and non-deacylated PIs (Peterson and Cummings, 2006). Reverse phase chromatography is a method that uses a hydrophobic stationary phase, which is produced by the alkyl chains covalently bonded to the solid support (3-5 μm silica beads) (Fig 1.13). Separation in reverse phase chromatography occurs due to the different binding properties of the solutes present in the sample, as a result of their differences in polarity. The use of a polar mobile phase allows the deacylated glycerated inositol head groups to be separated from the non-polar molecules and eluted out quickly from the column finally, forming a mass spectrum.

Fig 1.13: Reverse phase chromatography. Reverse phase chromatography consists of a hydrophobic stationary phase with a polar mobile phase allowing sufficient separation of hydrophilic molecules. The hydrophilic molecules elute out quicker. The stationary phase consists of C18 ligands, which are highly hydrophobic. This allows a strong interaction between non-polar molecules allowing polar molecules to pass through the column and produce a mass spectrum (Adapted from Peterson, 2006). This is used as a means of separating the polar heads from hydrophobic tails.

Fig 1.14: Mass Spectrum showing retention time of deacylated PI standards focusing on parent peaks of specific PIs (PtdIns: 333m/z, PtdInsP: 413m/z, PtdInsP₂: 493, PtdInsP₃: 573m/z). Stationary phase consist of a C18 column. Mobile A: H₂O(+5mM dimethylhexylamine (DMHA), solvent B: acetonitrile (+5mM DMHA). Gradient: 0.1%B to 50%B in 25 min. (extracted image from Kiefer *et al.*, 2010). More OH groups the molecule contains, the more polar it is, thus the faster it eluted out from the column as distinguished in this image. DMHA is used as an ion-pairing buffer.

There are two types of column solute elution systems that can be used in HPLC: a) isocratic flow and b) gradient elution. Isocratic elution is a separation method, where the mobile phase composition remains constant throughout the experiment. The gradient elution method involves a change in composition of the mobile phase during the separation of different molecules. Due to the various classes of PIs, choice of mobile phase is critical in order to help elute the derivatives at different times. Hence, a gradient elution is preferred over an isocratic flow. The mobile phase varies depending on the polarity of the analyte. The mobile phase of a gradient flow solvent can begin with a high polarity (water 90%: MeOH 10%), and gradually decrease in polarity eluting out the less polar molecules, as seen in Fig 1.14. Retention time (time it takes molecule to elute from column and reach mass detector) will vary for the various deacylated PIs due to the number of OH groups present on the inositol head. Once eluted from the column, the deacylated PtdIns are subjected to MS where a chromatogram is produced by the computer.

Lipid Detection

Ion Source: Electrospray Ionization (ESI)

Once the lipids are separated, they are subjected to MS through the ion source head. There are numerous types of ionization methods used such as electron ionization (EI), fast atom bombardment (FAB), matrix-assisted laser desorption ionization (MALDI), and electrospray ionization (ESI) (El-Aneed *et al.*, 2009).

Developed in 1989 by John Fenn, ESI transforms analytes in solution into ions in the gas phase (Fenn *et al.*, 1989; Bruins, 1991). ESI has been especially important for mass analysis of polar compounds such as biological polymers (for example, proteins, peptides,

glycoproteins, and nucleotides), pharmaceuticals and their metabolites; and industrial polymers (for example, polyethylene glycols) (Ho *et al.*, 2003). Essentially ESI is ideal for analysis of PIs due to its superior sensitivity, and with abilities to detect analytes at very low concentrations. (Zhu *et al.*, 2010).

The solvent carrying the analyte is eluted through the column, to the source head, passing through a narrow orifice, creating a spray to which a high voltage (3-5Kv) is applied (Fig 1.15). This process creates a fine mist of droplets that are charged at their surface. As the solvent evaporates, the electrical charge density reaches a critical point where the droplets divide into smaller droplets due to the electrostatic repulsion being greater than the surface tension (Ho *et al.*, 2003; Bruins, 1991). After this process is repeated numerous times, the highly charged ions are injected into the gas phase by electrostatic repulsion. Depending on the molecule of interest, the ESI probe can be used either in the positive or negative polarity mode. Negative ions have lost a proton and positive ions have gained a proton. Some of the factors that impact the quality of electrospray are droplet size, liquid surface tension, surface charge, ion solvation strength and finally solvent volatility.

Fig 1.15: Electrospray Ionization. A soft ionization technique used to determine the weights of proteins, peptides and biological macromolecules. The molecules are being ionized into smaller droplets where they are further desolvated into smaller droplets. These can be passed through mass analyzer to the detector where the samples can be analyzed (Finnigan™ LTQ™ Getting started manual).

Additionally, the type of solvent used can have a profound impact on the quality of electrospray. Solutions compatible with the MS are organic/aqueous solvents (methanol, acetonitrile (AcN), isopropyl alcohol), volatile acids (acetic acid, formic acid) and bases (ammonium acetate, ammonium formate) (Finnigan™ LTQ™ Getting started manual). The ions then pass through the ion transfer capillary where they are further detected based on their m/z ratio.

Mass Analyzer: Linear Ion Trap

Once the molecules of interest are ionized, they are further analyzed based on their m/z by a mass analyzer. The linear ion trap quadrupole mass filter manipulates direct current and radio frequency potentials in order to select and filter for the masses of interest (Fig 1.16). This was first described in 1950 by Paul Wolfgang (El-Aneed *et al.*, 2009).

Fig 1.16: Operation of a quadrupole mass analyzer. The ion (M^+) travels from the source, through the four metal rods arranged parallel to each other and finally reaching the detector (Adapted from El-Aneed *et al.*, 2009).

The quadrupole mass analyzer consists of four rods placed parallel to each other. Ions are separated based on their trajectories in the oscillating electric fields (Banerjee and Mazumdar, 2011). Only ions carrying a specific m/z will reach the detector after they pass down the length of the quadrupoles. The direct current (DC) and radio frequency (RF) voltages can be set so that the amplitudes of oscillation for the desired m/z ratio are stable with the ions travelling along the z -axis, reaching the detector. Ions carrying unstable trajectories will collide and fall off the rods not allowing them to reach the detector. Some of the major advantages of using a quadrupole mass analyzer are the low cost, small size, and the ease of maintenance (Banerjee and Mazumdar, 2011).

To obtain a substantial amount of structural information about a specific analyte, tandem MS/MS can be used where parent ions produced by the ion source are further fragmented by collision with helium (buffer gas) in the mass analyzer resulting in product ions (also known as collision-induced dissociation; CID; Jennings, 1968). This requires multiple mass analyzers to be connected in a series (Fig 1.17).

Fig 1.17: Representation of Tandem Mass Spectrometry (MS/MS). Simplest form of fragmentation in mass spectrometry is the ionization of the parent ion to form product ions. To obtain more detail information of the analyte of interest would be to combine two mass spectrometers. The first mass spectrometer is used to select a single (parent) ion that is a characteristic of a given analyte in a mixture. The mass-selected ions pass through a region where they are activated that causes them to fall apart to produce fragment (product) ions. This is done by colliding the ions with a neutral gas such as helium, process known as collision induced dissociation (CID). The second mass spectrometer is used to separate the fragment ions according to mass. The resulting MSMS spectrum consists only of product ions from the selected precursor or previous product ions. (Adapted from Banerjee and Mazumdar, 2011).

The first analyzer is responsible for the isolation of the ions, while the second analyzer (collision cell) is responsible for ion fragmentation, and finally the third separates fragmented ions based on their m/z values. This technique enables the user to obtain a considerable amount of structural information about the unknown molecule (Banerjee, 2011).

Computational Analysis of MS data

Following the mass analysis, a mass spectrum output yields a spectrum containing the fragment ion peaks (Fig 1.18). Peaks represent the ions that are formed in the mass spectrometer; the most intense of which is known as the base peak. Using the base peak as 100% abundance, the relative abundance of other ions is shown. Once a mixture of phospholipids is subjected to the mass spectrometer from a biological sample, it generates spectra with hundreds to thousands of peaks associated with the molecular ions present in the sample. In lipid extracts, these peaks represent ions from phospholipids, buffer ions and contaminants. Hence, it is vital to ensure precise purification and separation steps are completed prior to injection in order to limit the contaminant peaks. PIs as well as other lipids can be identified through their m/z values, or through their CID fragments providing a fingerprint for the PIs. Peaks can be further confirmed by using lipid databases such as Lipid Library, Lipid Bank, Lipidat, and Lipid Maps to name a few, as seen in Table 1.2. A comprehensive classification system for lipids provides an overview of these lipid molecules and their structures. The goal of these lipid databases is to characterize lipids and to identify new ones, to quantitate level changes of lipids within cellular metabolism and to establish dynamic lipid networks.

Fig 1.18: Chromatogram of a glycerated PI. Graphical representation of the relative abundance of ions intensity versus the m/z ratios

Quantitation of PIs

Once the structures of PIs have been identified, it is critical to quantitate the levels of PIs present within a normal cell versus a disease state cell. Initially, it is best to start off with an internal standard, which acts as a control for extraction efficiency in order to compensate for losses during sample preparation, low abundance PI species of interest and variable detection sensitivity of the MS. The structure of an ideal internal standard should resemble the analyte by its physiochemical properties, and in some practices, it is best to synthesize an internal standard by incorporating stable isotopes on the molecules of interest (Ho *et al.*, 2003). Currently, there are a limited number of internal standards available. Another factor that is important to consider is that biological samples give off significantly lower ionization signals compared to pure standard solutions with the same concentrations (Ho *et al.*, 2003). This could be due to the high concentrations of non-volatile materials from biological samples present in the sample subjected to the MS with the analyte. Thus, extensive purification processes are required, where HPLC is an ideal method to separate the non-volatile compounds from the analyte.

Once the sample is prepared, the conditions of analysis on MS need to be optimized in order to quantitate the deacylated PtdIns(4,5)P₂. Once optimal settings are found, concentrations of the biological samples subjected into the MS need to be considered. Very high concentrations show ion suppression effects. It has been shown that molecules with higher mass will suppress the signal of smaller molecules and that more polar analytes are more susceptible to suppression (Annesley, 2003). Serial dilutions (or using smaller

injection volumes) are made to ensure that the response lies on the linear part of the graph (Fig 1.19). If this is not the case, the response may be saturated or suppressed.

Fig 1.19: Range to achieve optimum results. Anywhere below or above the dotted lines, the intensity may be either too low or too high causing either too low of a signal or broad peaks and over saturation.

***S. cerevisiae* as a model organism**

Saccharomyces cerevisiae (fungus, yeast, eukaryote) is an ideal model organism for the study of PI's due to extensive research on lipids. Importantly, yeast functions and regulatory mechanisms appear co-related in humans. The lipid profiling in *S. cerevisiae* has been extensively researched with a plethora of mutant strains that affect PI concentration, such as Fig4 Δ (30-50% decrease in PtdIns(3,5)P₂, and Fab1 Δ (necessary for synthesis of PI(3,5)P₂) (Botelho et al., 2008).

Clinical Studies of PIs

Optimizing MS conditions to quantitate and differentiate between different PIs isomers is of extreme importance since PIs have been implicated in almost all aspects of cell physiology (Di Paolo and De Camillo, 2006). This research may lead to therapeutic

targets to treat diseases epidemic to the world (Pendaries, 2003; Nicot and Laporte, 2008). Metabolism of PIs is highly controlled. The strict regulation present within cellular signals allows the PI's to proceed with the normal functions within the cells. Any disruption or alterations in this signaling mechanism can lead to detrimental diseases. The discovery and the functional characterization of PI's (Dove *et al.*, 1997; Rameh *et al.*, 1997), PtdIns-binding proteins (Lemmon, 2003; Balla, 2005), and PI-metabolizing enzymes (Majerus *et al.*, 1999; Odorizzi *et al.*, 2000; Roth, 2004) have dramatically advanced the field of lipidomics due to the large role they play in regular and irregular cellular processes such as cell survival, apoptosis, phagocytosis, cancer, diabetes, cardiovascular and neurodegenerative diseases (Forrest *et al.*, 2013; Ghigo *et al.*, 2012; Bothmer and Jolles, 1994).

Objectives

Previous studies (Wakelam and Clarke, 2011) have shown the chromatographic resolution of PIs at their native structures. However, due to the complexity of these lipids, there are a number of issues that complicate PI analysis by MS including the very low concentration of the PIs relative to other phospholipids, most are protein bound making the extraction process more difficult, and due to their highly polar head groups, product recovery is limited. Due to these reasons, it has become very difficult to measure the levels of PIs using traditional quantitation methods. We hypothesize, that the procedure described below will provide high recovery of PtdIns(4,5)P₂, and when coupled with MSMS-ESI, we will be able to quantify PtdIns(4,5)P₂ from both wild type and *INP51* mutant *S. cerevisiae* yeast cells. This technique could be useful to help analyze cellular processes with more detail and target diseases such as cancer, obesity, and other neurodegenerative diseases. The goal is to progress towards development of a method to quantitate PIs through MS.

Specifically the aims of this study were to:

1. Prepare a method for the extraction and purification of lipids from Yeast cells
 - a. Extract and purify PIs using following techniques
 - i. Bligh and Dyer method
 - ii. Prepare and optimize conditions on the MS for the separation and quantitation of deacylated PIs
 - b. Sep-Pak cartridges (reverse phase chromatography)
 - c. Thin layer chromatography to confirm the presence of phospholipids
2. Quantitating the standard, Gro(4,5)P₂ in the wild type and mutant yeast cell extracts
 - a. Working with the Gro(4,5)P₂ standard (Echelon BioSciences)
 - i. Constructing a Standard Curve by using the MS
 - ii. Obtain the fragmentation pattern of this standard
 - b. Be able to use the Tune file (which is a file containing the optimum MS settings to check for the sensitivity and detection levels of deacylated PI(4,5)P₂) from the standard against the biological sample
 - i. Presence of the parent ion peak in selected ion monitoring (SIM) mode
 - c. Run the Biological sample with different dilutions and observe if the peak intensity increases/decreases depending on the different dilutions (SIM)
 - d. Compare wild type Gro(4,5)₂ levels to the mutant *INP51* yells. Mutant cells in this case are thought to have 2-4 times more PtdIns(4,5)P₂ compared to the wild type.

Chapter 2: Materials and Methods

Sample Preparation

Yeast Cell Growth

S.cerevisiae SEY 6210 yeast cells were grown in a yeast-peptone media (YP) overnight to an OD₆₀₀ of 0.6. The tubes were centrifuged (10⁷, 2, 500 rpm, 10 min) to pellet. Once the supernatant was discarded, 1mL of 1x yeast nitrogen base (YNB) was re-suspended with the pellet. The tubes were spun at 2,500 rpm for 10 min, supernatant discarded, and the pellet was re-suspended in 500 µL of selective drop-out (SD) media.

Yeast cell homogenization and PI extraction using Bligh and Dyer method

10⁷ yeast cells were centrifuged at 220×g, for 3 min to pellet cells. The supernatant was discarded and the cells were resuspended in 1 mL ice cold 0.25M methanolic HCl. The sample was transferred to a 2 mL glass homogenizer and fully homogenized. The sample was transferred to a 15 mL polypropylene tube. The remaining sample in the homogenizer was rinsed out with 2 mL 0.25M methanolic HCl/chloroform (1:1) and repeated with 3 mL chloroform. The sample was vortexed for 30 sec, followed by sonication in an ice-cold bath for 10 minutes. The sample was split into two layers via the addition of 1.5 mL split phase buffer (0.25M HCl, 2mM EDTA, 5mM tetrabutylammonium hydrogen sulphate (TBAHS) and 0.9% NaCl). The sample was vortexed for 30 seconds, sonicated in an ice bath for 5 minutes and incubated on ice for an additional 10 minutes. The sample was neutralized by centrifugation at 250×g for 5 min and transferred to a tube containing 1.5 mL 100 mM EDTA pH 6 and 1.5 mL TBAHS.

The samples were re-extracted by addition of 4 mL synthetic lower phase buffer.

The synthetic lower phase buffer was prepared by addition of 150 μ L water, 2 mL methanolic 0.25 M HCl, 4 mL chloroform and 1.5 mL 0.25 M HCl, 2 mM sodium EDTA, 5 mM tetrabutylammonium hydrogen sulphate, and 0.9% NaCl. The layers were allowed to separate and 4 mL of the lower phase was utilized in the experiment. The yeast sample combined with synthetic lower phase was vortexed, sonicated in an ice-cooled bath for 5 minutes and incubated on ice for 5 minutes. Following centrifugation at 250xg for 5 min, the lower phase was combined with the original lower phase, vortexed and centrifuged at 250xg for 5 min. The upper phase was discarded and lower phase transferred to a clean tube and dried in a speedvac evaporator. After drying, the tube was rinsed with 500 μ L chloroform/methanol/water (5:5:1) and dried in a speedvac evaporator.

Deacylation of PIs

While spinning, the deacylation reagent was prepared (deacylation reagent consisted of 4.6 mL methanol (MeOH), 2.6 mL methylamine (40% stock), 1.6 mL distilled water (ddH₂O), and 1.1 mL 1-butanol). The supernatant was aspirated, and the pellet was re-suspended in 50 μ L of ice-cold ddH₂O. This was transferred to new eppendorf tubes and 1000 μ L of deacylation reagent was added and the tubes were sonicated. The tubes were incubated at room temperature for 30 min, sonicated and incubated in a water bath at 53°C for 50 min. Once the deacylation step was completed, the tubes were vortexed to remove any material sticking to the walls and speed vac dried over night.

Extraction of deacylated PIs

Once the tubes were dried down, 500 μ L of ddH₂O was added and the tubes were sonicated to dispersion. Tubes were incubated at room temperature for 30 min and speed vac dried again. Once the samples were dried down, 300 μ L of ddH₂O was added and

sonicated to dispersion. Extraction reagent was made as follows: 4 mL 1-butanol, 800 μ L ethyl ether and 200 μ L of formic acid ethyl ester (ethyl formate). For the extraction process, 300 μ L of extraction reagent was added and the tubes were vortexed for 30 sec. The tubes were spun at maximum speed at room temperature. This formed a biphasic layer where the aqueous layer is at the bottom containing the deacylated inositol head groups. The bottom layer is pipetted out to a new tube. The extraction process is repeated once again added 300 μ L of the extraction reagent. Once the entire aqueous layer is transferred to a new tube, discard the remaining upper layer. The new tube containing the aqueous layer is dried down in a speed vac and re-suspended in 60 μ L of HPLC H₂O. The sample is vortexed and frozen at -20°C until analyzed by MS.

Confirmation of cellular lipid extracts with Thin Layer Chromatography (TLC)

Aluminum-backed silica gel TLC plates (silica gel 60F₂₅₄) purchased from Sigma, were used for the standard TLC separation of lipid samples. Before loading, the extracted lipid standards were spotted on the TLC plates, approximately 20 μ L of the running solvent to a standard glass TLC tank was added. The tank was sealed allowing the atmosphere inside the tank to equilibrate for at least 30 min. The running solvent was chloroform/methanol/4 M NH₄OH (54:42:12, v/v). At least 1.0 cm of space was left between each sample position. The dried lipid extracts (before deacylation) were re-suspended in 150-200 μ L chloroform: methanol (2:1). The samples were loaded in their pre-determined positions along a line drawn with a pencil 1.5 cm to 2 cm above the bottom of the plate. Once the spots were fully loaded, the plate was positioned in the glass tank, the tank was covered and the solvent was allowed to run up the TLC for a few min until the solvent front was within 2.5 cm of the top of the plate. After the plate dried, it was placed in

a jar containing iodine crystals. The iodine vapourized caused colour changes on the TLC plate, which signify particular molecular groups in the extracts such as inositol head groups, phosphate groups and glycerol groups.

Chromatography

Reverse-Phase Chromatography using the Sep-Pak cartridge

In order to perform reverse phase chromatography with the Sep-Pak column, a gradient of strong to weak polar solvent was used. This chromatography method uses less solvents and reagents; there is minimal sample transfer, no cross-contamination, and less exposure to toxic agents, which can eliminate isomerization of deacylated PIs. The Sep-Pak contains non-polar packing silica material. First, the bonded silica phase was solvated with 70% methanol (MeOH)+ an ion pairing reagent (0.1% NH_4OH), not allowing the cartridge to dry out. The wild type/mutant deacylated PI biological samples were dissolved in a strongly polar solvent. The deacylated PIs were dissolved in 70%MeOH+0.1% NH_4OH . The column was washed with water and eluted with acetonitrile (AcN) and then methanol (MeOH). The most tightly bound components were eluted with a more progressively non-polar solvent such as isopropanol (IPA), while the polar molecules of interest eluted out first. Once, the PIs were eluted in fractions, the cartridges were discarded. Each of the sample fractions were collected vials were collected and run through the MS to finally identify the fractions of interest. In this method, the sample is purified from any other contaminants.

HPLC Analysis of deacylated PIs

Two chromatography methods were tested using reverse phase chromatography. Samples were loaded onto a 2 μ L loop at a flow rate of 10 μ L/min. Before injecting, the samples were thawed. A standard curve was run in order to test for sensitivity and aid in the quantitation of PIs in biological samples. Standard curve was obtained with the Gro(4,5)P₂ (catalogue number: P-4500) ordered from Echelon Biosciences. The range for the standard curve consisted of the following contents: 0 pmol, 1 pmol, 5 pmol, 10 pmol, 15 pmol, 20 pmol, 30 pmol, and 40 pmol per 2 μ L. The dried biological samples were re-suspended in 70% MeOH + 0.1% NH₄OH and further diluted in 100% H₂O + 0.1% NH₄OH. The biological samples were diluted 1:1000, 1:100 and 1:10 so they did not over saturate the columns and cause any clogs within the ionization needle head. Deacylated PIs were analyzed under negative polarity. The 15 cm column used consisted of the following inner diameter: 320.1 μ m and outer diameter: 431.1 μ m. For reverse phase chromatography, the column was packed with 5 μ m C₁₈ silica beads. Ligands that are grafted on the silica beads were linear hydrocarbon chains (n-alkyl groups). This allowed the stationary phase to be highly hydrophobic. In this method, HPLC allowed molecules to be eluted at different times dependent on their molecular structures, hence decreasing the chances of ion suppression. Two methods consisting of various stationary and mobile phases were utilized for the detection system of PIs. Elution Method 1 (M1) consisted of a mobile phase A of 50% IPA and 50% MeOH + 0.1%NH₄OH, whereas, mobile phase B consisted of H₂O + 0.1%NH₄OH. Elution method 2 (M2) consisted of Mobile phase A of water containing 5mM N,N dimethylhexylamine (DMHA) and 4mM glacial acetic acid and mobile phase B of acetonitrile or methanol with 5mM DMHA and 4mM glacial acetic acid.

Cleaning the column

In order to clean the C₁₈ column for any impurities and contamination, 5% formic acid (FA) was injected through the column to elute out any non-polar bound molecules. Isocratic flow consisting of 70% MeOH was run for 30 min until all peaks present from the samples disappeared in negative polarity. All syringes and loops are cleaned with 70% MeOH or 5% formic acid (FA) before injection of any samples.

Chapter 3: Results

PIs regulate major aspects of cellular behavior such as trafficking, phagocytosis, apoptosis and cell survival. Comparing the lipidome of healthy versus diseased states can provide information helpful in correlating the role of lipids in various diseases, such as cancer, atherosclerosis, and chronic inflammation. With the measurement of PtdIns(4,5)P₂ using mass spectrometry, it can provide insight into such areas as drug/inhibitor interactions. Methodology based on deacylation of PIs and subjecting these to HPLC-MS can be used to quantitate PtdIns(4,5)P₂ in both wild type and mutant cell types. Various optimization steps were used to achieve optimum results for the detection of PtdIns(4,5)P₂. Both mobile phases were changed in order to achieve high resolution, enhance separation, decrease carry over and peak tailing and increase sensitivity. This work represents the first step to quantitate PIP₂ and other PIs.

Confirmation of the presence of extracted and deacylated products by TLC

TLC was used as a confirmation step to ensure there was something in the extracted and deacylated samples. This allowed us to verify if we extracted amphipathic lipids in one phase versus head groups in another phase, also a way to test the efficiency of the extraction and deacylation protocol. A screening mechanism was set in place in order to visualize and detect any product formed from the extraction and deacylation steps of the Bligh and Dyer and methylamine deacylation methods. This allowed the detection of specific molecular species present on phospholipids. The detection system consisted of iodine vapor, which has been previously used for the general visualization of phospholipids

in yeast cell extracts. The running solvent system consisted of MeOH: chloroform: 4M (NH₄)OH (54:42:12) (fig 3.1)(Berrie and Lurisci, 2007). We were able to confirm the certainty of the molecular identity of the extracts spotted on the stationary phase based on their molecular composition and polarity. Standard, biological samples after deacylation and biological samples before deacylation were spotted. The separation of the samples are depended on the hydrophobicity of the compounds. Since silica gel plates are polar, less polar compounds samples (full intact lipids) will migrate faster than the more polar (after deacylation, glycerated PIs), which was retained in the plate. These plated were then stained with iodine vapor detecting the presence of either inositol, phosphate and glycerol groups which appeared after 2 and 5 minutes. Both Gro(4,5)P₂ standard and biological deacylated PIs were spotted and the results state that since these samples are very polar, its presence was closer to the line of origin. Finally, biological samples before deacylation were run and its presence was detected closer to the solvent front line stating the less polar property of these intact lipids compared to the glycerated PI head groups. Due to the presence of potential phospholipid groups present, TLC confirmed the high efficiency of extracted lipids and deacylated PI groups thus, comparing the molecular results to the Gro(4,5)P₂ standard to confirm its presence or absence.

Fig A

Fig B

Fig 3.1. The iodine vapor detection mechanism consists of exposing the TLC plates to iodine vapor. First TLC plate has been exposed to iodine vapor for 120 seconds whereas Fig B plate has been exposed to iodine vapor for 480 seconds, because some functional groups require more time to be stained. The running solvent used was MeOH: chloroform: 4M (NH₄)OH, v/v (54:42:12). The deacylated (lane 1) and non-deacylated (lane 2) lipid extracts were dissolved in of 70% MeOH. Plate 1, lane 1 appeared to have a white blot near the point of application, row lane2 consisted of a light brown streak whereas row 3 consisted of a medium brown blot. Plate 2, lane 1 consisted of a very pale yellow spot, lane 2 consisted of a pale to medium yellow streak and lane 3 consisted of a medium brown-yellow streak and a brown blot near the top. The following represents colors for specific characteristic functional groups in the molecule: glycerol is medium yellow, glycerol phosphate is medium yellow, inositol is medium brown with a pale white center surrounded by a brown ring (Larose, 1961). This shows the efficiency of the extraction and deacylation protocol.

Recovery of PI(4,5)P₂ from the Sep Pak

Due to the very low levels of PtdIns(4,5)P₂ present within cells, the extraction steps need to be sufficient to provide optimum product recovery. To confirm recovery of Gro(4,5)P₂ from the solid-phase extraction method, eluents to recover majority of Gro(4,5)P₂ and the amount of Gro(4,5)P₂ recovered was measured.

Solvent system for the recovery of Gro(4,5)P₂ using the Sep Pak reverse phase column

Sep Pak cartridge is a convenient and reproducible solid-phase extraction device used in the final extraction process to purify a sample before it is injected into the MS as indicated by the manufacturers, Waters. The cartridges consisted of a few factors that makes it a convenient choice for extraction of the polar Gro(4,5)P₂: low cost, faster protocol compared to LLE, less sample handling, reduced harm to labile samples and most importantly greater percentage of product recovery, in comparison to other methods such as TLC, dry-column method and the Soxhlet extraction method.

Various solvent systems were used to elute the internal standard. They ranged from lower to higher polarity (AcN, isopropanol, isopropanol + 5% formic acid and finally water. Initially, a stock of 100 μ M Gro(4,5)P₂ was diluted 1:1000 and 1:00 to obtain parent peak of 493 m/z. Eluents' that provided peaks well above the back ground noise (E³) consisted of AcN and water. The final wash step with water over saturated the column, which lead to peak trailing as seen fig 3.2.

Fig 3.2: Recovery analysis of Gro(4,5)P₂ using the solid phase extraction procedure. Various eluents were tested in order to extract Gro(4,5)P₂ from the reverse phase Sep Pak cartridge. Most polar solvents such as AcN and water recovered Gro(4,5)P₂ from the cartridge.

Measuring recovered Gro(4,5)P₂ from the solid phase extraction method

Now, that we know which solvent system provided the most recovery from the cartridge, next step is to measure how much of Gro(4,5)P₂ are we losing through the solid phase extraction system. Both the wash with water and AcN steps were combined together. Dilutions consisting of 1:100 and 1:10 were analyzed, both from before and after the solid phase extraction system. There seems to be a 90.8% recovery of Gro(4,5)P₂ after eluting it through the sep pak cartridge as shown in Fig 3.3.

Fig 3.3: Measurement of the amount of Gro(4,5)P₂ standard from Echelon recovered after elution through the Sep Pak . Dilutions consisting of 1:100 and 1:10 of before and after Sep Pak recovery of standards were injected through a 2 μ L loop particularly looking at the SIM peak with a m/z of 493. There is a 90.8% recovery of Gro(4,5)P₂.

Structural analysis of Gro(4,5)P₂

In order to measure levels of PI(4,5)P₂, methylamine was used to deacylate the lipid, detaching the lipid chain, thus forming Gro(4,5)P₂. This chemical reaction is important because the structural analysis of the full structure of PIs lead to a highly complex picture, due to the varying lipid tails attached to the inositol head groups through the glycerol backbone. Though these specialized lipids comprise less than 1% of the cellular lipid

cohort, mass spectrometry data confirms the presence of Gro(4,5)P₂ in wild type and mutant yeast cells.

Selected Ion Monitoring of Gro(4,5)P₂

Mass spectrometry is also a micro analytical technique used for the qualitative assessment of a specific analyte. It can be used to selectively determine the elemental composition and some aspects of the molecular structure of a molecule. MS was used to confirm the molar mass of Gro(4,5)P₂, which is the parent molecule in mass spectrometry as seen in fig 3.4. To start, the MS must be tuned in order to ensure correct identification of the ion mass to charge ratio and provide optimum settings in order to detect the specific parent ion (Fig A.1). Mass spectrum obtained a common product-ion analysis for m/z of 493 (Fig 3.4).

Fig 3.4: Full MS of the standard, Gro(4,5)P₂. The full mass spectrum of the parent ion of Gro(4,5)P₂ consisted of a m/z of 493 [M-H]⁻. This was obtained under a negative-ion scan mode. In negative ion mode, [M-H]⁻ is typically formed by the abstraction of a proton by the OH⁻ present on the Gro(4,5)P₂ molecule, thus forming negative ions. Here, ion peak is the most intense due to the optimization of this peak through tuning of the MS.

Product-ion mass spectrum of Gro(4,5)P₂ was used to obtain the structure information. Selected ion monitoring (SIM) was achieved in figure 3.5. This is a single stage technique in which a single ion is monitored. Ions of interest (493m/z) are stored within the mass analyzer where they are then scanned to form a mass spectrum.

Fig 3.5: Selected Ion Monitoring of Gro(4,5)P₂. Analysis of the parent ion of 493m/z. Limited m/z charge is monitored as opposed to the full mass spectrum range.

Selected Reaction Monitoring

A unique feature of MS is its capacity to produce and detect fragments of the molecule that correspond to discrete groups of atoms of different elements that reveal structural features. The main fragmentation profile of 493m/z was achieved using negative scan mode by direct infusion and selecting for the parent peak. Selected reaction monitoring (SRM), is a two stage monitoring system, which was used to confirm the identity of Gro(4,5)P₂. Both the parent ions and product ions were monitored. The parent ion (493 m/z) and then was fragmented to form product ions. Peak consisting of a m/z of

395 was the most stable ion consisting of the Gro(4,5)P₂ without the addition of glycerol and a water molecule being formed as seen in figure 3.6.

Fig 3.6: Selected reaction monitoring of Gro(4,5)P₂. Fragmentation pattern of Gro(4,5)P₂ in which collision-induced dissociation is used as a means to increase selectivity. The parent ion is filtered (493 m/z) and fragmented to produce product ions of m/z of 177, 241, 321, 339, 395, 401, and 475. This helps reveal structural features of Gro(4,5)P₂. Y axis represents the relative abundance/intensity of the ion peak. X axis represents the mass mass-to-charge ratio (m/z) of the ion.

Methods of Detection

Various conditions were tested to identify and obtain the most intense and prominent peaks to develop a standard curve and then run biological samples. The diluted biological samples consisting of the deacylated head groups were compared to the standard curve in order to quantitate the amount of lipid of interest present in both the wild type and mutant yeast cell types. Two elution methods provided optimized results, however elution method 1 gave a very high backpressure (+300 bars) at a very low flow ~3 µL/min. Due to this, the peaks were broad and reaching baseline after each injection was

time consuming. M2 provided optimized results at a flow rate of $\sim 15 \mu\text{L}/\text{min}$, pressure at 200bars, and after intense peak height, the baseline was reached within 5 minutes.

Negative ion LC-MS spectra were recorded after optimization settings; under ion charge control conditions at a scan speed of $200,000\text{m}/\text{z}/\text{s}$. Spectra was recorded in a range of 100-600 m/z. Ammonium hydroxide was used as a ionization buffer. The standard curve was run to obtain two orders of magnitude. The limit of detection for the standard curve was to the point of over saturation and peak broadening. After the standard curve is run, several blank samples were run until no carry-over was noted. A $2 \mu\text{L}$ loop was used to inject the samples in the MS. Blanks were run initially to obtain a stable base line. Pico mole amounts were detected using the ESI-MS. A $100 \mu\text{M}$ stock was used of Gro(4,5) P_2 . Blanks were run after the standard curve series to stabilize baseline. Once the baseline stabilized, 1:100 or/and 1:10 dilution of the biological sample was injected providing peaks within range of the standard curve and well above the blank injections. Elution method 1 and elution method 2 consisted of the similar stationary phase, which was C_{18} silica bound beads.

Results of Elution Method 1

Mobile phase A consisted of 50% IPA and 50% MeOH + 0.1% NH_4OH , whereas, mobile phase B consisted of H_2O + 0.1% NH_4OH . Both SIM and SRM were run as seen in figure 3.4 and 3.55, respectively.

A standard curve using SIM was obtained having a range of 0, 1, 5, 10, 15, 20, 30, 40 pmole content within a $2 \mu\text{L}$ loop. In fig 3.7, three blanks were run after the 74 min period. Interestingly, carry over was significantly reduced compared to previous methods using i.e. mobile phase consisting of 70% AcN, and all were blanks are consistent in intensity of

approximately $9.11\text{E}+03$. After establishing a standard curve with the standard Gro(4,5)P₂ purchased from Echelon Biosciences, yeast biological samples were then run with varying dilutions of 1:100 and 1:10. There seemed to be a nearby to 3-fold increase in the intensity relative to the wild type of the 1:10 dilution of the deacylated wild type phosphoinositides in the biological sample.

Fig 3.7: SIM analysis of Gro(4,5)P₂ using elution method 1. The increasing intensity of the parent ion peaks (493 m/z) is correlated with the increasing content of Gro(4,5)P₂ per μL . A signal was present and was within the standard curve range.

We also established a standard curve using the fragment of 395 derivative by SRM. Product ion consisting of a m/z ratio of 395 was used for routine quantitation and the confirmation of PI(4,5)P₂, primarily due to its high stability after fragmentation. The same

content range was used as used in the SIM method: 0, 1, 5, 10, 15, 20, 30, 40 pmole of Gro(4,5)P₂ per 2 µL loop. Various dilution ranges of the wild type biological were used for the biological sample however no signal intensity was detected which ranged over the background noise as seen in fig 3.8. Dilution ranges consisted of the following: 1:1000, 1:100, 1:10, 1:5, 1:2 and 1:1. There was no signal detected for the wild type biological samples.

Fig 3.8: SRM analysis of Gro(4, 5)P₂ using elution method one. SRM was obtained using the product ion of 395 m/z. The biological peak at 395 m/z did not appear over a number of dilutions after the 120 min mark period. The biological wild type signal seems to be concealed with the background noise. The mobile phase consisted of the following; 50% mobile phase A and 50% mobile phase B. Used elution method One.

Results for elution method 2

Elution method 2 provided lower backpressure of approximately 200 bars at a flow rate of 15 $\mu\text{L}/\text{min}$ whereas elution method 1 consisted of a back pressure well above 300+ bars. Mobile phase A consisted of water containing 5mM N,N dimethylhexylamine (DMHA) and 4mM glacial acetic acid and mobile phase B of acetonitrile or methanol with 5mM DMHA and 4mM glacial acetic acid. A standard curve was run as described in fig 3.9 with the sequential injection of both deacylated lipids from wild type and mutant right after had a dilution of 1:100 and 1:10.

Fig 3.9: Using elution method 2, SIM of Gro(4,5)P₂ using method 2. Pmole amounts were injected; blank, 1pmol, 5, 10, 15, 20. Blanks were injected again to obtain a stable baseline. Biological wild type and mutant was diluted 1:100 and 1:10. The mutant provided a 2 fold increase in comparison to the wild type.

The standard curve of the standard Gro(4,5)P₂ without the blank provided a linear relationship between the intensity and the content of Gro(4,5)P₂. The r value consisted of 0.9912, stating that there was a strong correlation between both the intensity and the content of Gro(4,5)P₂ injected in the 2 µL loop (fig 3.10).

Fig 3.10: Relationship between intensity and the content of Gro(4,5)P₂ in a 2µL loop. With the deduction of the blank from the standard curve, the standard curve provided a strong correlation between both the x and y values, also confirming the high sensitivity of the MS due to the linear relationship.

Confirmation of structure by analyzing the retention times

In order to confirm and compare the chemical composition of a particular analyte, a 10 min gradient was run (Table 3.1) to observe the retention time of the Gro(4,5)P₂ standard and the mutant Gro(4,5)P₂ (fig 3.11).

Time	%B
0	5
1	5
1.01	12.5
7	70
8	70
9	5
10	5

Table 3.1: Using elution method 2, 10 min gradient used to elute Gro(4,5)P₂. The polarity of the eluent increases gradually with time helping the most polar molecules elute. A gradient of mobile phase B started off with 5%B increasing to 12.5%B to 1.01 min, it progressively increased to 70% by 6.99 min and back down to 5%B in 1 min and remained as the 10 min gradient finished.

Fig 3.11: Retention time of the standard Gro(4,5)P₂ using the SIM method. With the gradient outline in Table 3, the Gro(4,5)P₂ ion of 493 m/z standard seemed to have started eluting out of the reverse phase C₁₈ column at 2.5 min.

Next, the retention time for the biological Gro(4,5)P₂ was observed and compared to that of the standard Gro(4,5)P₂ (fig 3.12). There seemed to be a few seconds (58 seconds) difference between the two elution of the standard compared to the biological Gro(4,5)P₂.

Fig 3.12: Retention time of the wild type biological Gro(4,5)P₂ using the SIM method. The biological Gro(4,5)P₂ parent ion of 493 m/z started eluting out at 1.8 min. A trail was left due to the over saturation of the biological sample injected into the reverse phase C₁₈ column.

With various methods used for the confirmation of the presence of the parent ion of 493 m/z ratio, there seems to be Gro(4,5)P₂ detected in both the wild type and mutant yeast cells. Elution method 2 seemed to provide the most optimum results in comparison to other methods used for the measurement of Gro(4,5)P₂.

Chapter 4: Discussion:

PIs play a fundamental role in diverse functions within the cell. They are known to play a role in cell survival and differentiation, opening of ion channels, calcium mobilization, serve as precursors of intracellular signaling cascades and as ligands for membrane associated proteins that are involved in trafficking and cytoskeletal dynamics (Wenk *et al.*, 2003; Wakelam and Clark). The changes in different levels of PIs, is the hallmark of various mutants. There are various efforts to explore abnormal lipid metabolism in human diseases, such as insulin-resistant diabetes, Alzheimer's disease, schizophrenia, cancer, atherosclerosis, and infectious diseases through lipidomics approach (Kim *et al.*, 2010). With the focus on phosphoinositide, lipidomics can contribute to drug development for pharmaceutical therapy of phosphoinositide related diseases. Advances in separation and quantitation technology have changed the analysis of biological samples, intensely. Previous attempts at quantitation of PIs have focused on utilizing [3H]-inositol or [32P] inorganic P² radiolabelling of cell cultures, followed by lysis, extraction, deacylation and resolution using TLC or HPLC. The radioisotopes are then quantified by a liquid scintillation counter. This approach is disadvantageous as it is laborious since it required a permit to be used, time-consuming and utilizes radioactivity, which requires special handling care. Labeling occurs only during turnover, thus newly synthesized PIs are labeled but the preexisting PIs are not; thus unaccounted for in the quantitation. Additional assays have been developed based on enzymatic activity and interaction domains, which also lack sensitivity. Recently a shift towards MS, which has previously been utilized to quantify low abundance peptides, metabolites and lipids is occurring.

Due to the over complexity of PIs, different methods are employed for the separation and analysis of different classes. Since the importance lies on the phosphorylation pattern on the inositol head group to different PIs, the fatty acid chain was deacylated in order to simplify MS analysis. PIs were extracted by using the Bligh and Dyer method, where a biphasic phase is formed. One phase contained polar deacylated molecules whereas the other phase nonpolar un-deacylated lipids and fatty acids. The advantage of using the Bligh and Dyer method over the Folch method, which is another common phospholipid extraction method, is the reduction in the solvent/sample ratio. Bligh and Dyer uses one part sample to three parts solvent whereas, Folch method employs a ratio of one part sample to twenty parts solvent (Iverson *et al.*, 2001). Despite this, the Bligh and Dyer method is thought to yield recovery of almost 95% of total lipids (Bligh and Dyer, 1959). Even though his method has been long used in the extraction of phospholipids, many have used modified versions of these methods for the extraction of PIs and reduce product loss. Pettit *et al.*, used a method which is thought to recover approximately 90% of PIs (Pettitt, 2010). This procedure is believed to provide high extraction recovery of all PIs from biological samples, thus identifying the lipid structures by CID of the MS peaks. To confirm the presence of phospholipids in the cellular extract, a TLC was run to identify the chemical composition of the extract such as certain molecular groups common to phospholipids. There are many advantages to using TLC for lipid analysis. TLC is convenient, simple, inexpensive, consumes smaller amounts of solvent than HPLC, enables the analysis of several samples in parallel, and it is easy to visualize by stains or dyes such as iodine vapour to specifically characterize certain functional groups (Fuchs *et al.*, 2011). Referring to Figure 3.1, deacylated PIs blot is closer to the line of origin versus

PIs extracted with the Bligh and Dyer method before deacylation thus, confirming the difference in polarity of the two. This step is important because it confirms the presence of extracted phospholipids and deacylated species of PIs within the recovered samples from yeast cells. Then the contents of the biological sample were run through a Sep pak cartridge in order to eliminate contaminants that might otherwise co-found in HPLC-MS analysis. The cartridge did not seem to have significant loss of Gro(4,5)P₂ as seen in figure 3.3 because the recovery rate was approximately 91%. This step is vital because without the use of this cartridge, it can clog the column leading to new ion suppression (A.2).

The quantitation of Gro(4,5)P₂ was made by constructing a standard curve using commercial Gro(4,5)P₂ with SIM analysis (Fig 3.7). The quantification of the concentration of an analyte with MS analysis employs a correlation between the content and the ion intensity of the analyte, which shows a linear dynamic range as seen in fig. Different amounts of Gro(4,5)P₂ were spiked to reach contents from 0.5 pmol/μL to 10 pmol/μL before the presence of over saturation and peak broadening. This enabled us to test for the sensitivity of the MS which allowed us to detect down to 0.5 pmole/μL amounts of Gro(4,5)P₂. Overall, the construction of the standard curve validates that the linear dynamic range of quantification is present in the SIM scan mode (Fig 3.10). Hence, validating the accuracy and sensitivity of MS for measurement of specific analytes. Other deacylated PIs can also be used in order to measure their concentration within both wild type and mutant cell types. Accordingly, results can be validated by the accuracy of the two-step quantification procedure utilizing the combination of both full MS scan and MS/MS scans. Hence, when using the SRM mode for the quantification of the biological

derived Gro(4,5)P₂, it can become problematic due to reasons, such as the non-successful chemical deacylation reactions and low recovery of deacylated PIs.

Another confirmation step to compare the chemical composition of the standard Gro(4,5)P₂ and the biological Gro(4,5)P₂ is to run a gradient, analyzing the retention time for both samples. Both the standard (Fig 3.11) and the biological derived Gro(4,5)P₂ (fig 3.12) eluted out at similar time points; the standard eluted out at 1.9 min whereas the biological derived Gro(4,5)P₂ eluted out at 2.9 min. Thus, stating that the polarity of two is similar. The slight difference could be due to the presence of other head groups in biological derived samples. These results are similar to that of Kiefer *at al.*, because most polar deacylated species elute with a solvent containing high polarity.

Two types of yeast cells were compared, wild type SEY 6210 and *INP51* mutants (fig 3.9). The wild type have steady-stated levels of PtdIns(4,5)P₂ that can change upon stimulation or disruption of genes thus, in order to account for changes in the level of these, it is vital to compare it to cells with a few fold increase or decrease in levels of PtdIns(4,5)P₂. Inositol polyphosphate 5-phosphatase (5-ptase) enzyme comprise a family of critical proteins that remove the 5-position phosphate from either soluble or lipid inositol polyphosphates, or both. The yeast genome sequence has similar open reading frames to mammalian inositol signaling genes. One reading frame of importance is YIL002C, designated here as *INP51*, is known to be highly similar to 5-ptases (Stolz, 1998). The gene *INP51* encodes a 108-kDa protein which has intrinsic PtdIns(4,5)P₂ 5-ptase activity (Stolz, 1998). Any loss or gain of function of *INP51* demonstrates that 5-ptase activity is required for proper PtdIns(4,5)P₂ maintenance (Stolz, 1998). We used *S. cerevisiae* cells lacking *inp51* mutants, which have a 2-4 increase in levels of PtdIns(4,5)P₂

and Ins(1,4,5)P₃. This gene is critical for PtdIns(4,5)P₂ homeostasis and cold tolerant growth. Cells over expressing *inp51* exhibit a 35% decrease in levels of PtdIns(4,5)P₂. Thus, proper function of *INP51* is required for proper *in vivo* maintenance of this PI (Stolz *et al.*, 1998). Both wild type and mutant PtdIns(4,5)P₂ levels were compared by injecting dilutions of 1:100 and 1:10 of deacylated lipid extracts. There seems to be a strong difference between 1:10 wild type and 1:10 mutant Gro(4,5)P₂ within the cells consisting of a 2.08 fold in *INP51* mutant cells in comparison to the wild type *S. cerevisiae* cells. The dilutions consisting of 1:100 seemed to not have a correlation with the 1:10 dilution, which could be due to the very small concentration of PI(4,5)P₂ present within the cells. Hence, the results vary when trying to measure this deacylated phospholipid at such low concentrations. This could be because the cellular lipidome is a very complicated system, potentially comprised of hundreds of thousands of individual lipid molecular species (Yang and Han, 2012). PIs are known to be less abundant lipids in cells; therefore the analysis of these PIs is limited and the replication of these results varies depending on sample treatment. Caution must be taken during each step, as product loss is very common during the extraction and deacylation steps. Also, the ion intensity of an analyte measured with MS could be easily affected by other minor alterations in the conditions of analyte ionization and instrumentation and therefore quantification results may vary or may be irreproducible at a fixed concentration of an analyte (Yang and Han, 2012). Due to this, measurement of Gro(4,5)P₂ with MS can be compared to another standard that has a similar structure to Gro(4,5)P₂ such as Gro(3,4)P₂. This analyte can then be analyzed simultaneously with Gro(4,5)P₂ to ensure there is a similar ratio of loss of product over various runs. This will allow us to analyze accordingly and distinguish loss of PI's in cells.

Chapter 5: Conclusions and Future Work

In conclusion for the first time, the methodology described in this paper, allows for a rapid, sensitive and comprehensive analysis of PI(4,5)P₂ in yeast cells which is a great benefit in the study of these molecules in their many roles. A standard purchased from Echelon Biosciences was used to generate a standard curve and to compare the measurement of biological PI(4,5)P₂ in yeast cells. For the biological samples, using ESI-MS, the detection limit of Gro(4,5)P₂ seemed to be 1 pmol in wild type cells, relevant to other studies where the detection limit of common PIs is between 1-10pmol. Several solvent mixtures and addition of modifiers were tested. Replacing AcN by MeOH enabled the optimum quantitation of PI(4,5)P₂ in yeast cells. Replacing elution method 1 by elution method 2, and decreasing column size from 15 cm to approximately 10cm, allowed lower back pressure for the analysis of PI(4,5)P₂. To test the applicability of our method to biological samples, we analyzed mutant type yeast cell extracts that are known to produce 2-4 fold increased amount of PI(4,5)P₂ compared to the wild type due to its INP51 gene deletion genotype. Our method clearly succeeded in reproducing the increased amounts of PI(4,5)P₂ in mutant yeast cell types. Three replicates of both wild type and mutant were run independently, in order to validate empirical data and limit error. The mutant biological Gro(4,5)P₂ levels were on average 3.9 fold higher than that of the wild type. However, while relative changes in PI(4,5)P₂ were possible to detect, quantitation of the absolute content of PI(4,5)P₂ was not reproducible as seen in A3. This could be due to the loss of PI(4,5)P₂ from the several extraction and deacylation steps, employed. The method presented here was successfully applied to *s. cerevisiae* in terms of approximate

measurement of PI(4,5)P₂. The extraction procedure of all PIs remains a major concern due to poor recovery rates, which decrease with increasing phosphorylation.

PIs are an important class of cellular components and play diverse roles in human physiology. Even though, they are very important, there has been limited progress upon analyzing these lipids in detail, both in wild type mutant cell types. MS is a powerful technique which allowed the measurement of Gro(4,5)P₂ for both mutant and INP51 wild type yeast cells. Even though MS has the ability to detect Gro(4,5)P₂, there are still a few key problems in PI analysis such as, extraction, stability and recovery while at the same time being able to measure the different levels of PIs present within the cell. There has to be specific modifications reached during the extraction and recovery process, particularly on PIs in order to have a better understanding of what their function is within cells and how they may affect other molecular models. For future work, enzymatic biological reaction can be used to catalyze the formation of Ins(1,4,5)₃ and DAG from PI(4,5)P₂. Here, Ins(1,4,5)₃ can be used for analysis purposes. Another way to account for loss of PIs is to use the precursor PtdIns for measurement of PI(4,5)P₂, since levels of PtdIns should not change within wild type and INP51 mutant yeast cell types. Over various runs, the ratio of PtdIns versus the ion of interest can be accounted for to quantitate the loss of the specific deacylated PI. With the advent of MS technology, if the measurement of PIs can be achieved with optimum recovery, lipidomics have the key to cure to many disorders such as abnormal lipid metabolism in human diseases, such as diabetes, Alzheimer's disease, schizophrenia, and cancer to name a few.

Chapter 5: Appendix

Fig A.1 Gro(4,5)P₂ Tune File. A tune file is created automatically by the MS, in order to optimize the specific peak of interest.

Fig A.2: Running biological samples before Sep Pak. Running a series of dilutions of biological samples before eluting the sample through a Sep Pak cartridge clogs the needle.

Trial	Mutant/Wild type Ratio
1	2.08
2	1.25
3	8.42

Table A.1: Three replicates of biological and mutant derived Gro(4,5)P₂. The results were not reproducible for all three replicates, however the relative intensity was similar for two runs.

(a) Organic Compounds

(b) Functional groups

(c) Linkages in biochemical compounds

Fig A.3: Important Functional groups that may be found in various lipids and chemicals for deacylation and extraction of lipids. A)organic compounds, b) functional groups, c)linkages in biochemical compounds.

Saturated

Unsaturated

Fig A.4: Fatty Acid chains found in lipids. Unsaturated (single bond) fatty acid chain and saturated (double bond) found in fatty acids.

	Trial 1	Trial 2	Trial 3
Wild type	2.89E2	1.04E4	3.24E4
Mutant	1.27E3	2.5E4	6.5E4

Table A.2: Intensity for three replicates of Biological derived Gro(4,5)P₂. Intensity compared to standard curve of wild type and mutant INP51 biological derived Gro(4,5)P₂

Trial 1 pm	Trial 2	Trial 3	Mean	Standard deviation
0.2	14	49	21.067	25.15578131
1.4	20	99.68	40.36	52.20763163

Table A.3: P mol Content for three replicates of Biological derived Gro(4,5)P₂. Content in p mol for three replicates of wild type and mutant INP51 biological derived Gro(4,5)P₂

Chapter 6: References

- Annesley, T.M. 2003. Ion suppression in mass spectrometry. *Clin Chem* 49:1041-1044.
- Balla, T. 2005. Inositol-lipid binding motifs: signal integrators through protein-lipid and protein-protein interactions. *J Cell Sci* 118:2093-2104.
- Berrie, C., Lurisci, C. 2007. Analysis of PIs and their aqueous metabolites. *Methods in Enzymology* 434
- Bligh, E.G., and W.J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 37:911-917.
- Brugger, B., G. Erben, R. Sandhoff, F.T. Wieland, and W.D. Lehmann. 1997. Quantitative analysis of biological membrane lipids at the low picomole level by nano-electrospray ionization tandem mass spectrometry. *Proc Natl Acad Sci U S A* 94:2339-2344.
- Bruins, A.P. 1991. Liquid chromatography-mass spectrometry with ionspray and electrospray interfaces in pharmaceutical and biomedical research. *J Chromatogr* 554:39-46.
- Botelho, R.J., J.A. Efe, D. Teis, and S.D. Emr. 2008. Assembly of a Fab1 phosphoinositide kinase signaling complex requires the Fig4 phosphoinositide phosphatase. *Mol Biol Cell* 19:4273-4286.
- Clarke, N.G., and R.M. Dawson. 1981. Alkaline O leads to N-transacylation. A new method for the quantitative deacylation of phospholipids. *Biochem J* 195:301-306.
- Cullen, P.J., G.E. Cozier, G. Banting, and H. Mellor. 2001. Modular phosphoinositide-binding domains--their role in signalling and membrane trafficking. *Curr Biol* 11:R882-893.
- Cutler, R.G., J. Kelly, K. Storie, W.A. Pedersen, A. Tammara, K. Hatanpaa, J.C. Troncoso, and M.P. Mattson. 2004. Involvement of oxidative stress-induced abnormalities in ceramide and cholesterol metabolism in brain aging and Alzheimer's disease. *Proc Natl Acad Sci U S A* 101:2070-2075.
- Di Paolo, G., and P. De Camilli. 2006. Phosphoinositides in cell regulation and membrane dynamics. *Nature* 443:651-657.
- Dove, S.K., F.T. Cooke, M.R. Douglas, L.G. Sayers, P.J. Parker, and R.H. Michell. 1997. Osmotic stress activates phosphatidylinositol-3, 5-bisphosphate synthesis. *Nature* 390:187-192.

Dove, S.K., R.C. Piper, R.K. McEwen, J.W. Yu, M.C. King, D.C. Hughes, J. Thuring, A.B. Holmes, F.T. Cooke, and R.H. Michell. 2004. Svp1p defines a family of phosphatidylinositol 3, 5-bisphosphate effectors. *The EMBO Journal* 23:1922-1933.

Dowler, S., R.A. Currie, D.G. Campbell, M. Deak, G. Kular, C.P. Downes, and D.R. Alessi. 2000. Identification of pleckstrin-homology-domain-containing proteins with novel phosphoinositide-binding specificities. *Biochemical Journal* 351:19.

Downes, C.P., A. Gray, and J.M. Lucocq. 2005. Probing phosphoinositide functions in signaling and membrane trafficking. *Trends Cell Biol* 15:259-268.

Ejsing, C.S., E. Duchoslav, J. Sampaio, K. Simons, R. Bonner, C. Thiele, K. Ekroos, and A. Shevchenko. 2006. Automated identification and quantification of glycerophospholipid molecular species by multiple precursor ion scanning. *Anal Chem* 78:6202-6214.

Fahy, E., S. Subramaniam, H.A. Brown, C.K. Glass, A.H. Merrill, Jr., R.C. Murphy, C.R. Raetz, D.W. Russell, Y. Seyama, W. Shaw, T. Shimizu, F. Spener, G. van Meer, M.S. VanNieuwenhze, S.H. White, J.L. Witztum, and E.A. Dennis. 2005. A comprehensive classification system for lipids. *J Lipid Res* 46:839-861.

Fenn, J.B., M. Mann, C.K. Meng, S.F. Wong, and C.M. Whitehouse. 1989. Electrospray ionization for mass spectrometry of large biomolecules. *Science* 246:64-71.

Folch, J., M. Lees, and G.H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* 226:497-509.

Fuchs, B., R. Suss, K. Teuber, M. Eibisch, and J. Schiller. 2011. Lipid analysis by thin-layer chromatography--a review of the current state. *J Chromatogr A* 1218:2754-2774.

Garcia, P., R. Gupta, S. Shah, A.J. Morris, S.A. Rudge, S. Scarlata, V. Petrova, S. McLaughlin, and M.J. Rebecchi. 1995. The pleckstrin homology domain of phospholipase C-delta 1 binds with high affinity to phosphatidylinositol 4,5-bisphosphate in bilayer membranes. *Biochemistry* 34:16228-16234.

Gericke, A., M. Munson, and A.H. Ross. 2006. Regulation of the PTEN phosphatase. *Gene* 374:1-9.

Gillooly, D.J., A. Simonsen, and H. Stenmark. 2001. Cellular functions of phosphatidylinositol 3-phosphate and FYVE domain proteins. *Biochemical Journal* 355:249.

Gunnarsson, T., L. Ekblad, A. Karlsson, P. Michelsen, G. Odham, and B. Jergil. 1997. Separation of polyphosphoinositides using normal-phase high-performance liquid chromatography and evaporative light scattering detection or electrospray mass spectrometry. *Anal Biochem* 254:293-296.

Han, X., and R.W. Gross. 2003. Global analyses of cellular lipidomes directly from crude extracts of biological samples by ESI mass spectrometry a bridge to lipidomics. *Journal of lipid research* 44:1071-1079.

Han, X., and R.W. Gross. 2005. Shotgun lipidomics: electrospray ionization mass spectrometric analysis and quantitation of cellular lipidomes directly from crude extracts of biological samples. *Mass Spectrom Rev* 24:367-412.

Harkewicz, R., and E.A. Dennis. 2011. Applications of mass spectrometry to lipids and membranes. *Annu Rev Biochem* 80:301-325.

Ho, C.S., C.W. Lam, M.H. Chan, R.C. Cheung, L.K. Law, L.C. Lit, K.F. Ng, M.W. Suen, and H.L. Tai. 2003. Electrospray ionisation mass spectrometry: principles and clinical applications. *Clin Biochem Rev* 24:3-12.

Hurley, J.H., and T. Meyer. 2001. Subcellular targeting by membrane lipids. *Curr Opin Cell Biol* 13:146-152.

Ivanova, P.T., S.B. Milne, M.O. Byrne, Y. Xiang, and H.A. Brown. 2007. Glycerophospholipid identification and quantitation by electrospray ionization mass spectrometry. *Methods Enzymol* 432:21-57.

Iverson, S.J., S.L. Lang, and M.H. Cooper. 2001. Comparison of the Bligh and Dyer and Folch methods for total lipid determination in a broad range of marine tissue. *Lipids* 36:1283-1287.

Jennings, K. 1968. Collision-induced decompositions of aromatic molecular ions. *International Journal of Mass Spectrometry and Ion Physics* 1:227-235.

Kiefer, S., J. Rogger, A. Melone, A.C. Mertz, A. Koryakina, M. Hamburger, and P. Kuenzi. 2010. Separation and detection of all phosphoinositide isomers by ESI-MS. *J Pharm Biomed Anal* 53:552-558.

Kim, Y., S.R. Shanta, L.H. Zhou, and K.P. Kim. 2010. Mass spectrometry based cellular phosphoinositides profiling and phospholipid analysis: a brief review. *Exp Mol Med* 42:1-11.

Kimber, W.A., L. Trinkle-Mulcahy, P.C.F. Cheung, M. Deak, L.J. Marsden, A. Kieloch, S. Watt, R.T. Javier, A. Gray, and C.P. Downes. 2002. Evidence that the tandem-pleckstrin-homology-domain-containing protein TAPP1 interacts with Ptd (3, 4) P2 and the multi-PDZ-domain-containing protein MUPP1 in vivo. *Biochemical Journal* 361:525.

Krauss, M., and V. Haucke. 2007. Phosphoinositide-metabolizing enzymes at the interface between membrane traffic and cell signalling. *EMBO Rep* 8:241-246.

Larose, J.A. 1961. The use of Iodine Vapor as a general detecting agent in the thin layer chromatography of Lipids. *The Journal of the American Oil Chemists Society* 39:

- Larose, J.A. 1961. The use of Iodine Vapor as a general detecting agent in the thin layer chromatography of Lipids. *The Journal of the American Oil Chemists Society* 39:
- Lemmon, M.A. 2003. Phosphoinositide recognition domains. *Traffic* 4:201-213.
- Majerus, P.W., M.V. Kisseleva, and F.A. Norris. 1999. The role of phosphatases in inositol signaling reactions. *Journal of Biological Chemistry* 274:10669-10672.
- Martin, T.F. 1998. Phosphoinositide lipids as signaling molecules: common themes for signal transduction, cytoskeletal regulation, and membrane trafficking. *Annu Rev Cell Dev Biol* 14:231-264.
- Milne, S.B., P.T. Ivanova, D. DeCamp, R.C. Hsueh, and H.A. Brown. 2005. A targeted mass spectrometric analysis of phosphatidylinositol phosphate species. *J Lipid Res* 46:1796-1802.
- Nicot, A.S., and J. Laporte. 2008. Endosomal phosphoinositides and human diseases. *Traffic* 9:1240-1249.
- Odorizzi, G., M. Babst, and S.D. Emr. 2000. Phosphoinositide signaling and the regulation of membrane trafficking in yeast. *Trends Biochem Sci* 25:229-235.
- Pacetti, D., M. Malavolta, F. Bocci, E. Boselli, and N.G. Frega. 2004. High-performance liquid chromatography/electrospray ionization ion-trap tandem mass spectrometric analysis and quantification of phosphatidylcholine molecular species in the serum of cystic fibrosis subjects supplemented with docosahexaenoic acid. *Rapid Commun Mass Spectrom* 18:2395-2400.
- Pendaries, C., H. Tronche, M. Plantavid, and B. Payrastre. 2003. Phosphoinositide signaling disorders in human diseases. *FEBS letters* 546:25-31.
- Pentchev, P.G., M.E. Comly, H.S. Kruth, M.T. Vanier, D.A. Wenger, S. Patel, and R.O. Brady. 1985. A defect in cholesterol esterification in Niemann-Pick disease (type C) patients. *Proc Natl Acad Sci U S A* 82:8247-8251.
- Perona, J.S., and V. Ruiz-Gutierrez. 2003. Simultaneous determination of molecular species of monoacylglycerols, diacylglycerols and triacylglycerols in human very-low-density lipoproteins by reversed-phase liquid chromatography. *J Chromatogr B Analyt Technol Biomed Life Sci* 785:89-99.
- Peterson, B.L., and B.S. Cummings. 2006. A review of chromatographic methods for the assessment of phospholipids in biological samples. *Biomed Chromatogr* 20:227-243.
- Pettitt, T.R. 2010. Phosphoinositide analysis by liquid chromatography-mass spectrometry.

Methods Mol Biol 645:203-217.

Pettitt, T.R., S.K. Dove, A. Lubben, S.D. Calaminus, and M.J. Wakelam. 2006. Analysis of intact phosphoinositides in biological samples. *J Lipid Res* 47:1588-1596.

Postle, A.D., D.C. Wilton, A.N. Hunt, and G.S. Attard. 2007. Probing phospholipid dynamics by electrospray ionisation mass spectrometry. *Prog Lipid Res* 46:200-224.

Pulfer, M., and R.C. Murphy. 2003. Electrospray mass spectrometry of phospholipids. *Mass Spectrom Rev* 22:332-364.

Rameh, L.E., A. Arvidsson, K.L. Carraway, A.D. Couvillon, G. Rathbun, A. Crompton, B. VanRenterghem, M.P. Czech, K.S. Ravichandran, and S.J. Burakoff. 1997. A comparative analysis of the phosphoinositide binding specificity of pleckstrin homology domains. *Journal of Biological Chemistry* 272:22059-22066.

Rusten, T.E., and H. Stenmark. 2006. Analyzing phosphoinositides and their interacting proteins. *Nat Methods* 3:251-258.

Sasaki, T., S. Takasuga, J. Sasaki, S. Kofuji, S. Eguchi, M. Yamazaki, and A. Suzuki. 2009. Mammalian phosphoinositide kinases and phosphatases. *Prog Lipid Res* 48:307-343.

Schneider, R., and G. Daum. 2006. Analysis of yeast lipids. *Methods Mol Biol* 313:75-84.

Shisheva, A. 2003. Regulating Glut4 vesicle dynamics by phosphoinositide kinases and phosphoinositide phosphatases. *Front Biosci* 8:s945-s967.

Stefan, C.J., A. Audhya, and S.D. Emr. 2002. The yeast synaptojanin-like proteins control the cellular distribution of phosphatidylinositol (4,5)-bisphosphate. *Mol Biol Cell* 13:542-557.

Stenmark, H., R. Aasland, and P.C. Driscoll. 2002. The phosphatidylinositol 3-phosphate-binding FYVE finger. *FEBS Lett* 513:77-84.

Stolz, L.E., W.J. Kuo, J. Longchamps, M.K. Sekhon, and J.D. York. 1998. INP51, a yeast inositol polyphosphate 5-phosphatase required for phosphatidylinositol 4,5-bisphosphate homeostasis and whose absence confers a cold-resistant phenotype. *J Biol Chem* 273:11852-11861.

Sturley, S.L., M.C. Patterson, W. Balch, and L. Liscum. 2004. The pathophysiology and mechanisms of NP-C disease. *Biochim Biophys Acta* 1685:83-87.

Thomas, A.E. 1964. Quantitative Estimation of Isomeric Monoglycerides by Thin-Layer Chromatography. *The Journal of the American Oil Chemists Society* 42:

Venkateswarlu, K., P.B. Oatey, J.M. Tavar[√]©, and P.J. Cullen. 1998. Insulin-dependent translocation of ARNO to the plasma membrane of adipocytes requires phosphatidylinositol 3-kinase. *Current biology* 8:463-466.

Wakelam, M.J., and J. Clark. 2011. Methods for analyzing phosphoinositides using mass spectrometry. *Biochim Biophys Acta* 1811:758-762.

Watt, S.A., W.A. Kimber, I.N. Fleming, N.R. Leslie, C.P. Downes, and J.M. Lucocq. 2004. Detection of novel intracellular agonist responsive pools of phosphatidylinositol 3, 4-bisphosphate using the TAPP1 pleckstrin homology domain in immunoelectron microscopy. *Biochemical Journal* 377:653.

Wenk, M.R. 2005. The emerging field of lipidomics. *Nat Rev Drug Discov* 4:594-610.

Wenk, M.R., L. Lucast, G. Di Paolo, A.J. Romanelli, S.F. Suchy, R.L. Nussbaum, G.W. Cline, G.I. Shulman, W. McMurray, and P. De Camilli. 2003. Phosphoinositide profiling in complex lipid mixtures using electrospray ionization mass spectrometry. *Nat Biotechnol* 21:813-817.

Yang, K., Han, X. 2012. Accurate quantification of lipid species by electrospray ionization mass spectrometry-meets a key challenge in lipidomics. *Metabolites* 1:21-40.

Ye, K., and J.Y. Ahn. 2008. Nuclear phosphoinositide signaling. *Front Biosci* 13:540-548

Zhainazarov, A.B., and B.W. Ache. 1999. Effects of phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 4-phosphate on a Na⁺-gated nonselective cation channel. *J Neurosci* 19:2929-2937.