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CHARACTERIZATION OF LYSOPHOSPHATIDIC ACID SUBSPECIES USING A NOVEL  
HPLC ESI-MS/MS METHOD

A Thesis submitted in partial fulfillment of the requirements for the degree of Masters of  
Science at Virginia Commonwealth University.

by

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University of Virginia, B.S. Chemistry, 2007

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## List of Abbreviations

AT	Acyltransferase
ATX	Autotaxin
BD	Bligh-Dyer
DAGK	Diacylglycerol kinase
EDG	Endothelial differentiation gene
FBS	Fetal bovine serum
fmol	Femtomole
GPCR	G-protein coupled receptors
HPLC	High-performance liquid chromatography
HPLC ESI-MS/MS	High-performance liquid chromatography electrospray ionization tandem mass spectrometry
LPA	Lysophosphatidic acid
LPC	Lysophosphatidylcholine
lyso-PLD	Lysophospholipase D
$m/z$	Mass-to-charge ratio
MALDI	Matrix-assisted laser desorption ionization
MAGK	Monoacylglycerol kinase
MRM	Multiple reaction monitoring
MS/MS	Tandem mass spectrometry
PA	Phosphatidic acid
PLA	Phospholipase A
PLA1	Phospholipase A1

PLA2	Phospholipase A2
PLD	Phospholipase D
pmol	Picomole
SPE	Solid phase extraction
TLC	Thin layer chromatography
TOF	Time-of-flight

## **Abstract**

# **CHARACTERIZATION OF LYSOPHOSPHATIDIC ACID SUBSPECIES USING A NOVEL HPLC ESI-MS/MS METHOD**

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Lysophosphatidic acid (LPA) is a bioactive lipid with a plethora of biological functions, including roles in cell survival, proliferation, and migration. Although high-performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC ESI-MS/MS) technology has been used to measure the levels of LPA in human blood, serum and plasma, current methods cannot readily detect the minute levels of LPA from cell culture. In this study, a

novel HPLC ESI-MS/MS method with enhanced sensitivity was developed which allows accurate measurements of LPA levels with a limit of quantitation at approximately 10 femtomoles. The method was validated by quantitation of LPA levels in the media of previously characterized cell lines ectopically expressing autotaxin. Autotaxin overexpression induced an increase in several subspecies of LPA while others remained unchanged. Lastly, this HPLC ESI-MS/MS method was validated via biological assays previously utilized to assay LPA production. Hence, this new HPLC ESI-MS/MS will allow researchers to measure *in vitro* LPA levels and also distinguish between specific LPA subspecies for the delineation of individual biological mechanisms.

## Introduction

### 1.1 Discovery of the Biological Activity of LPA

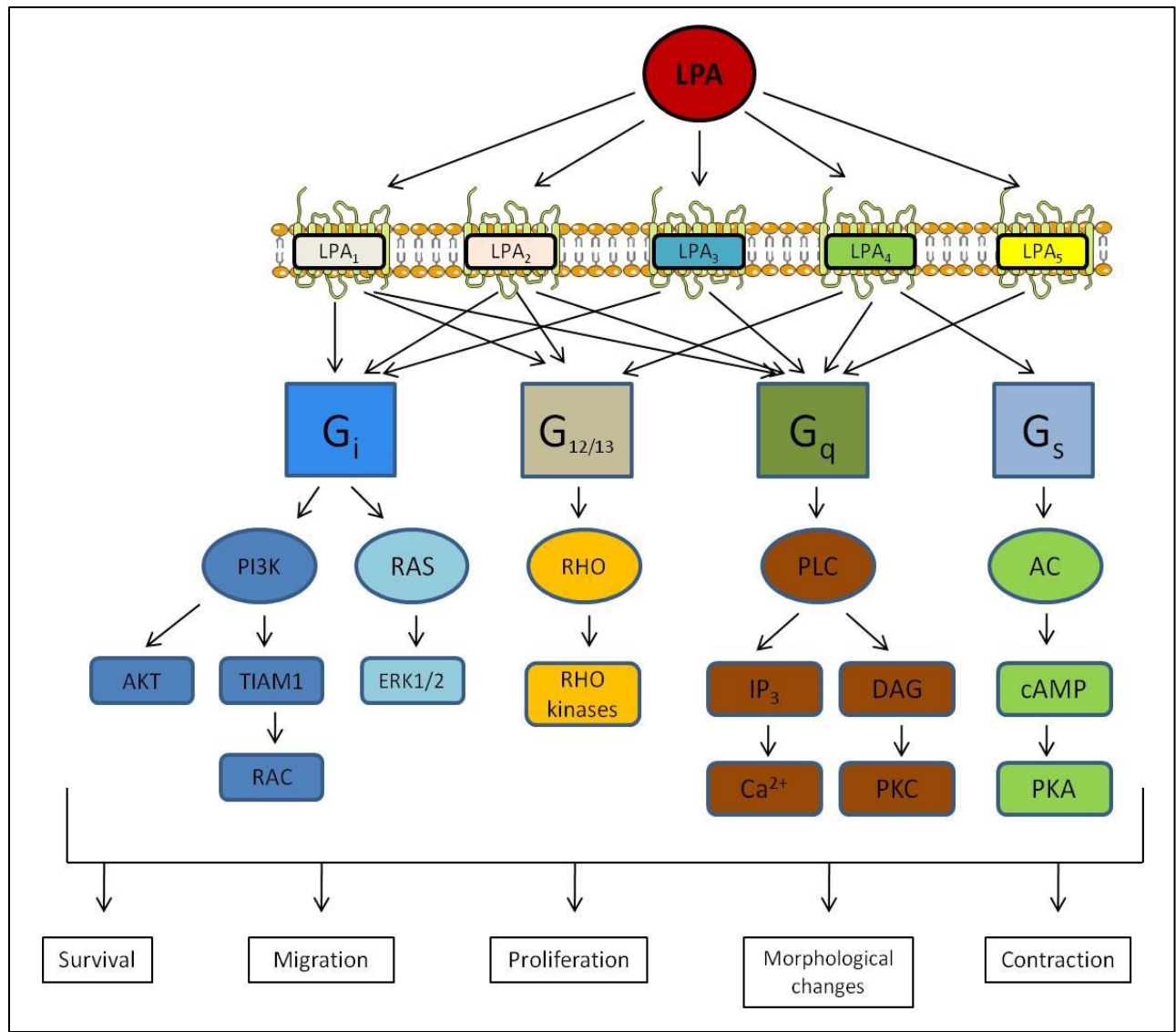
Lysophosphatidic acid (LPA) is a glycerophospholipid is composed of a single, variable length acyl chain, a glycerol backbone and a phosphate head group.<sup>1</sup> LPA had been known as a key lipid precursor for several decades, but the importance of LPA as a signaling molecule was not discovered until the mid-1980s. It was at this time when exogenous LPA was found to be the best and most potent  $\text{Ca}^{2+}$  mobilizing agonist and mitogen for quiescent fibroblasts.<sup>2</sup> In 1990, Jalink *et. al.*, showed that LPA concentrations in the low nanomolar range were sufficient to initiate the mobilization of  $\text{Ca}^{2+}$  across the plasma membrane.<sup>3</sup> These studies showed the growth-factor-like characteristics of LPA and that LPA had characteristics of a receptor ligand in that it was active well below its critical micelle concentration and displayed a dose-response relationship.<sup>2,3</sup> The study also demonstrated that the functions of LPA were cell-type specific, could not be recapitulated by other glycerolipids, and that LPA acted on the outer portion of the plasma membrane.<sup>2,3</sup> These observations led to the conclusion that extracellular LPA interacts with specific receptors on the plasma membrane to initiate downstream signaling cascades.

### 1.2 LPA Receptors

In 1996, the first G-protein coupled receptor (GPCR) specific for LPA was discovered.<sup>4</sup> In this study, Hecht *et. al.* showed the overexpression of the *vsg-1* gene resulted in increased cell rounding when treated with serum or LPA alone but did not increase when treated with other phospholipids.<sup>4</sup> This lead to the classification of the protein product of *vsg-1* as a LPA specific GPCR and the subsequent designation of this protein as  $\text{LPA}_1$ . Shortly thereafter, two other LPA GPCRs were discovered based on sequence homology and were subsequently named  $\text{LPA}_2$  and

LPA<sub>3</sub>.<sup>5-7</sup> LPA<sub>1-3</sub> are classified as members of the 'endothelial differentiation gene' (EDG) family. More recently, two additional GPCR specific for LPA have been discovered and named LPA<sub>4</sub> and LPA<sub>5</sub>.<sup>7-9</sup> LPA<sub>4</sub> and LPA<sub>5</sub> only share roughly 20% sequence homology to the EDG family receptors and are more closely related to the members of the purigenic receptor family. Therefore, they have been classified as members of the 'purinergic' (P2Y) receptors.<sup>10</sup> Although the LPA receptors have varying sequence homologies, they are all Type1, rhodopsin-like GPCRs that contain seven transmembrane alpha helices and couple to distinct heterotrimeric G-protein subtypes.<sup>7</sup> It is currently believed that there may be up to four more LPA specific receptors, but more research is necessary to properly characterize these receptors. The distinct coupling of the established LPA receptors and G-protein subtypes is depicted in Figure 1.

All of the established LPA receptors play important physiological roles. By activating GPCRs, LPA initiates the downstream signaling cascades depicted in Figure 1. These signaling cascades have important physiological functions that are outlined in Table 1 and Figure 1. As these figures clearly demonstrate, LPA plays a variety of important roles that affect normal biological functions. Since LPA plays many vital roles in human physiology, the dysregulation of LPA production or binding ability to LPA receptors can result in a variety of pathological conditions, a sampling of which are listed in Table 2.



**Figure 1:** LPA Receptors and Signaling Pathways

System	Phenotype	Roles for LPA signaling
Immune	Dendritic cell function	Maturation, chemotaxis
	T cell functions	Chemotaxis, apoptosis, trafficking, cytokine production
Vascular	Vasoregulation	Hypertension, endothelial cell death, loss of vascular integrity
	Vasculogenesis, Angiogenesis	Vasculature maintenance
Reproductive	Embryo implantation	Timing and spacing of implantation
	Spermatogenesis	Sperm motility, survival factor for germ cell
Nervous	Growth/development	Proliferation and differentiation of neural progenitor cells, neuronal survival, astrocyte proliferation, neurogenesis
	Morphology	Synapse formation, morphological changes in neurons and astrocytes
	Myelination	Differentiation of oligodendrocytes, Schwann cell proliferation, survival, and morphological changes

**Table 1:** Physiological Roles of LPA Signaling (adapted from <sup>11</sup>)

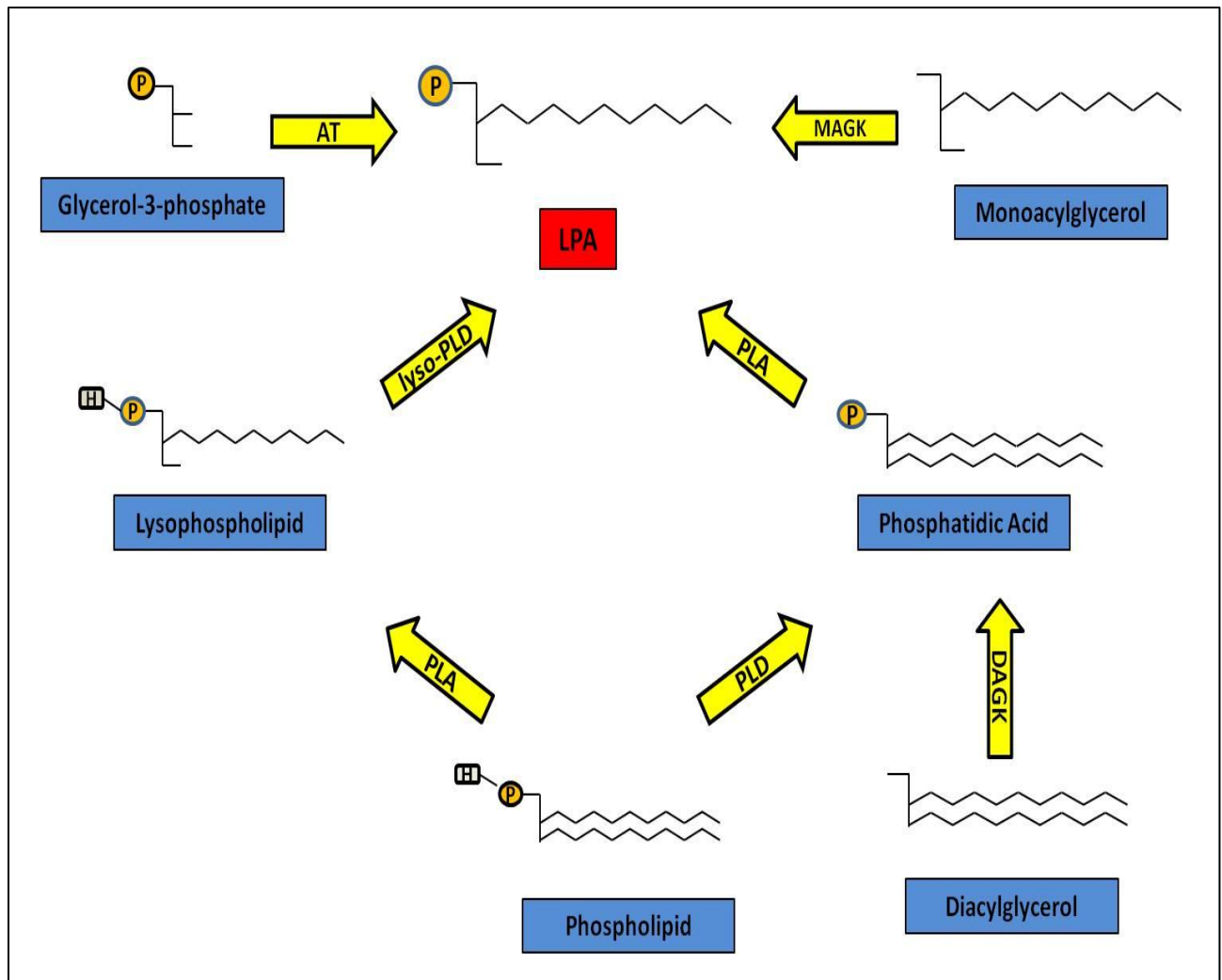


Human pathological conditions associated with LPA dysregulation	
Nerve injury	Pulmonary fibrosis
Neuro-inflammation	Renal fibrosis
Schizophrenia	Hepatic fibrosis
Developmental delay	Impaired wound healing
Bipolar disorder	Atherosclerosis
Cancer invasion and migration	Obesity
Osteoarthritis	Asthma

**Table 2:** Pathophysiological Conditions Associated with LPA Dysregulation (adapted from <sup>11</sup>)

### 1.3 LPA Synthesis

There are several methods of LPA synthesis (Figure 2), but the major pathways of LPA production are the cleavage of an acyl chain from phosphatidic acid (PA) via a phospholipase or the removal of a choline from lysophosphatidylcholine (LPC) by autotaxin.<sup>12-14</sup> PLA1 can cleave an acyl chain from the *sn*-1 position of PA to produce LPA while PLA2 can cleave an acyl chain from the *sn*-2 position of PA to produce LPA.<sup>13</sup> Although PLA1 and PLA2 can synthesize LPA, the major source of LPA production is the cleavage of a choline group from LPC by the enzyme autotaxin. Autotaxin is synthesized a prepro-enzyme that is proteolyzed by furin-mediated cleavage and secreted as an activated glycoprotein.<sup>15,16</sup> Originally identified as an autocrine motility factor secreted by melanoma cells, the discovery of the lyso-PLD function of autotaxin led to a better understanding of LPA production and function.<sup>15, 17-19</sup> The generation of LPA by autotaxin occurs by the hydrolysis of the choline group at the *sn*-3 position of lysophosphatidylcholine (LPC). The human gene that encodes autotaxin, *ENPP2*, contains 27 exons and has been shown to form three alternatively spliced products.<sup>16,20</sup> ATX $\alpha$ , also referred to as ATX $m$ , is a 915 amino acid protein that lacks exon 21 from *ENPP2* gene that was originally cloned from human melanoma cell line A2058.<sup>21</sup> ATX $\beta$ , also referred to as ATX $t$ , is a 863 amino acid protein that lacks both exons 12 and 21 from *ENPP2* gene and was originally reported in human tetracarcinoma cells.<sup>22</sup> A third, less prominent isoform, ATX $\gamma$ , also known as PD-1 $\alpha$ , lacks exon 12 and seems to be brain-specific.<sup>23</sup> While the ability of autotaxin to produce LPA has been widely established, the specific LPA species produced by autotaxin isoforms is not well understood. Furthermore, the differential biological functions for the individual LPA subspecies have not been examined, mainly due to a lack of reliable method to analyze and quantitate LPA subspecies *in vitro*.



**Figure 2:** Routes of LPA Production. (H - head group, P - phosphate)

## 1.4 Lipid Extractions

Due to their wide variety of characteristics, no single extraction technique is sufficient for the separation of lipids from biological samples. Extractions protocols should be optimized to achieve most accurate and highest recovery for the individual lipid of interest. The classical lipid extraction protocols are liquid-liquid extractions in which lipids separate into the more apolar solvent. The most common lipid extraction technique was originally devised by Folch, *et. al.*, in 1956 and involves a liquid-liquid extraction using a 2:1 ratio of chloroform:methanol.<sup>24</sup> Most lipids, due to their hydrophobic character, will separate into chloroform phase since it is less polar than the methanol phase. Bligh and Dyer developed an adaptation of the Folch method to reduce solvent consumption with the separation again based on the polar character of the solvents.<sup>25</sup> The Bligh-Dyer technique utilizes a chloroform:methanol:water extraction in the ratio of 1.25:2.5:1 with a centrifugation step to aid in phase separation. Lipids will again separate into the less polar, chloroform layer.

Numerous adaptations have been made to the Folch and Bligh-Dyer methods for the extraction of particular lipid classes. One such adaptation was made by Merrill *et. al.*, for efficient extraction of sphingolipids. This method utilizes a single phase chloroform:methanol:water solvent combination and an overnight incubation at 48°C in order to account for the differing polarity among sphingolipids.<sup>26</sup> Although lipid-specific adaptations of the Folch and Bligh-Dyer methods have been the common standard for multiple generations, many other lipid extractions have recently been popularized, including supercritical fluid extractions, single solvent extractions, and solid phase extractions (SPE).

Supercritical fluids are gases that approach the density of liquids but have the ability to diffuse like a gas. Due to its apolar character, supercritical carbon dioxide has been used

efficiently for the extraction of lipids.<sup>27</sup> Although supercritical liquid extractions are environmentally friendly and can be easily established with simple laboratory apparatus, the extraction protocol has yet to become a widely used method of lipid extraction. Single solvent extraction techniques using solvents such as methanol, ethyl acetate, acetonitrile, and methyl *tert*-butyl ether have gained popularity because of the simple dilution and centrifugation protocol they entail.<sup>28</sup> These extractions, however, may not provide significant lipid recovery from samples that contain very low levels of the lipid being analyzed, particularly *in vitro* samples. SPE extractions are based on principles of column chromatography where sample is separated based on its interaction with a stationary phase. SPE lipid extractions typically entail silica-based columns as stationary phases in order to separate lipids from the more hydrophilic molecules based on the hydrophobic interactions with the stationary phase.<sup>29</sup> While SPE can provide remarkable recovery of certain lipid classes, the necessity of a separate SPE column for each sample can become economically unfeasible. As detailed above, no single extraction is sufficient for all lipids and therefore, several techniques should be tested for the optimal extraction of the specific lipid of interest.

## **1.5 Lipidomics**

Numerous detection and quantitation methods have been utilized for lipid analysis. After lipids have been extracted from samples, they must be further separated for accurate analysis. A classical technique for lipid separation is thin layer chromatography (TLC), a method that separates compounds based on capillary action in response to their interaction with the stationary phase.<sup>30</sup> High-performance liquid chromatography (HPLC) has replaced TLC as the preferred method of lipid separation in recent generations.<sup>29</sup> HPLC is similar in principle to TLC, as both

techniques separate lipids based on interaction with a stationary phase, but HPLC has proven to be a more versatile and more efficient method of separation. One advantage of HPLC separation of lipids is the ability to quickly and easily utilize different types of stationary phases for different categories of separation. In normal phase HPLC, lipids are separated based on head group interaction with a polar stationary phase and elution with a solvent gradient shifting from apolar to polar. This combination of stationary phase and solvent conditions allows for effective separation of lipid classes based on the charge of the molecule. Reverse phase chromatography separates lipids using apolar stationary phase and a solvent gradient that shifts from polar to apolar. This allows for accurate separation of lipid species based on the degree of hydrophobic interaction of the sample with an apolar stationary phase. The combination of normal and reverse phase HPLC in tandem allows for even greater discrimination of lipids based first on the charged head group and then on hydrophobic acyl chain length.<sup>31</sup> The flexibility, increased sensitivity, and greater technical ease have led to HPLC becoming the preferred method of lipid separation.

Following extraction and separation, lipids can be detected using ultraviolet detection, immunoassays, radiolabelling, and mass spectrometry. If a lipid contains a chromophore, such as a conjugated double bond system, it can be measured using ultraviolet detection. In this detection method, chromatographic solvents selected must not absorb in the ultraviolet range. Although ultraviolet detection can be extremely sensitive, its limitations include the fact that it is more qualitative than quantitative in nature and the limitations on chromatographic solvent conditions.<sup>32</sup> Immunoassays for specific lipid species are used, but are limited because they are not a direct detection method, are time-consuming and expensive, and are not available for all lipids. Radiolabelling using  $^{32}\text{P}$  has long been a common method of lipid measurement.<sup>33</sup> TLC or

HPLC coupled to a radiation detector has been effectively used to analyze lipids, but radiolabelling is labor intensive and is of a more qualitative than quantitative nature.

The rapid advances in mass spectrometric technology in the past two decades have revolutionized the quantitative analysis of lipids. Mass spectrometry provides analysis of compounds based on the mass-to-charge ratio ( $m/z$ ) of charged particles. The general schematic of mass spectrometric analysis of lipids proceeds in the following manner: the sample of interest is ionized and vaporized, then separated based on  $m/z$  by electromagnetic fields. The ions are then detected and processed to produce mass spectra that are used to provide accurate analysis. Although there are many instrument variations of mass spectrometers, the equipment necessary consists of a ion source, a mass analyzer, and a detector. The ion source is responsible for producing ions from the vaporized sample which then proceed to the mass analyzer for sorting by electromagnetic fields. A detector then measures the amount of ions present to produce a mass spectrum.<sup>31</sup>

There are several ionization sources and mass spectrometer combinations commonly used for lipid analysis. Matrix-assisted laser desorption ionization coupled to a time-of-flight spectrometer (MALDI-TOF) is one method commonly used for lipid analysis.. During MALDI-TOF analysis, samples are mixed with a chemical matrix and dried onto a plate. A laser is then used to ionize the sample, and the ions are introduced into a TOF mass spectrometer. The TOF mass spectrometer measures the amount of time it takes from injection until the ions reach the detector to produce a mass spectrum. Although MALDI-TOF provides very high sensitivity and rapid sample analysis, matrices can often produce high background signal, making quantitative measurements unreliable.<sup>34</sup> MALDI-TOF is therefore used primarily for qualitative analysis.

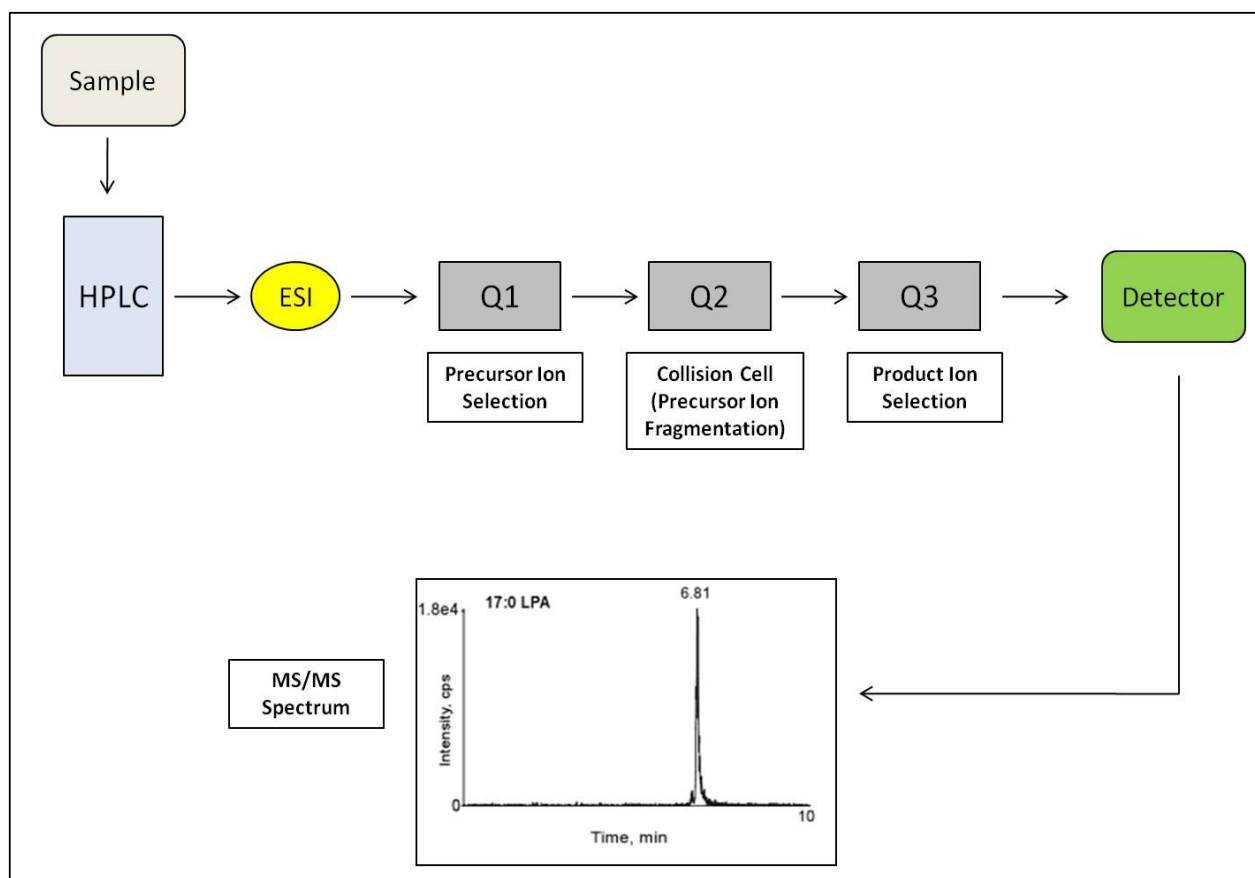
The most widely regarded methods for quantitative lipid analysis are direct injection into a mass spectrometer, also called shotgun lipidomics, or injection into a mass spectrometer following HPLC separation.<sup>31</sup> A shotgun lipidomics approach involves a direct injection of sample into the mass spectrometer, commonly using a syringe which is placed into a syringe pump to introduce the sample into the spectrometer at a steady rate in order to provide a steady signal.<sup>29</sup> The sample then undergoes mass spectrometric analysis as outlined in the preceding paragraph. While shotgun lipidomics is simple and quick, it is inappropriate for many samples because it may cause mass spectrometer contamination, signal reduction, and ion interference. It also does not efficiently separate lipid species and causes other issues that would make quantitative analysis of specific species unreliable. Thus, shotgun lipidomics has primarily been used as a screening tool for identification of lipids in a sample rather than for quantitation of lipid species.<sup>34</sup>

The preferred method of lipid quantitation is injection into a mass spectrometer following HPLC separation.<sup>29</sup> Samples undergo extraction and HPLC separation as detailed previously and are introduced into the mass spectrometer. This provides a sample which contains few contaminants and thereby increases sample throughput for the instrument. It also provides the ability to distinguish lipid species based on the retention time on the HPLC column.<sup>31</sup> Coupling of HPLC to a mass spectrometer capable of performing tandem mass spectrometric analysis has become the common standard for accurate lipid quantitation. Tandem mass spectrometry (MS/MS) is method of analysis that detects compounds based on the principle of measuring the transition of a precursor ion of interest to a product fragment. A schematic demonstrating HPLC ESI-MS/MS using a triple quadrupole mass spectrometer in multiple reaction monitoring mode (MRM) is depicted in Figure 3. In this method of lipid quantitation, HPLC separation is



performed and then the sample is introduced into the mass spectrometer. Ionization is performed by an electrospray source which then focuses a tight beam of ions for progression into the Q0 quadrupole. The ions are taken via vacuum into the Q0 quadrupole where they are trapped for progression into the Q1 quadrupole mass filter. The Q1 quadrupole scans and selects for a precursor ion. The selected precursor ions progress from Q1 quadrupole mass filter into the Q2 collision cell where they are fragmented to create a product ion that is selected in the Q3 mass filter. These product ions are then measured by the detector, and a spectrum is generated.

MS/MS uses the measurement of the distinct transition from precursor to product ion to provide accurate quantitation for compounds of interest. By selecting the transitions corresponding to individual lipid species and subspecies, HPLC ESI-MS/MS provides the most sensitive and accurate lipid quantitation method commonly used in modern research.



**Figure 3:** HPLC ESI-MS/MS Diagram

## 1.6 HPLC ESI-MS/MS Analysis of LPA

A variety of HPLC ESI-MS/MS methods have been developed to undertake LPA measurements from tissue, blood, plasma, and serum samples, but most are specific for separation and detection of LPA with little to no application in the detection of other lipid classes. Additionally, most of these methods demonstrate poor quality peaks or inadequate separation.<sup>35,36</sup> Some methods have even required pre-separation of LPA using thin-layer chromatography.<sup>37,38</sup> There are also methodological issues for the extraction of LPA in these reported protocols as most have relied on adapted Bligh-Dyer techniques with the use of very low pH conditions, which may lead to the degradation of LPC to LPA and provide artificially inflated LPA measurements.<sup>36, 39</sup> Even after acidic extractions, the limit of quantitation for LPA using these mass spectrometric methods have been in the low picomole or high femtomole range.<sup>37,39</sup> While this sensitivity is adequate for quantitation for the high LPA levels of *in vivo* biological samples, greater sensitivity as well as a reliable, non-volatile pre-separation and accurate extraction protocol are required for *in vitro* LPA measurements.

## 1.7 Project Objective

The objective of this study was to develop a HPLC ESI-MS/MS method for *in vitro* LPA quantitation while avoiding the induction of artificially enhanced LPA levels from acidic extraction protocols. Additionally, the separation and detection utilized for LPA was designed for capability with the separation and detection of many other lipid classes. The method was validated for *in vitro* use by demonstrating increased levels of LPA in autotaxin overexpressing ovarian cancer cell lines. The biological role of these increased LPA levels was further validated using previously established proliferation and migration assays for measuring the levels of this

bioactive lipid. This novel HPLC ESI-MS/MS method will provide researchers with the ability to measure *in vitro* LPA levels as well as provide a more sensitive and accurate method for LPA quantitation in biological samples.

## **Materials and Methods**

### **2.1 Materials**

SKOV3-Zeo, SKOV3-ATX, DOV13-Zeo, and DOV13-ATX cell lines were established by transfection of SKOV3/DOV13 cells with pcDNA3.1/Zeo or pcDNA3.1/Zeo-ATX (kindly provided by J Aoki, Kawasaki Medical School, Okayama, Japan) The transfected cells were selected with zeocin (500 ng/ml). Individual zeocin-resistant colonies were isolated by ring cloning and expanded sequentially in 24-well, 6-well and 60-mm plates. Expression of ATX protein was confirmed by immunoblotting. The ATX-positive clones were maintained as stable lines in RPMI (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 2% penicillin/streptomycin (BioWhittaker). SKOV3-Zeo and SKOV3-ATX clonal cell lines were cultured under 5% CO<sub>2</sub> at 37°C with routine passage every 2-3 days. LPA standards (14:0, 16:0, 17:0, 18:0, 18:1, 20:4) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Ammonium formate (Fluka) were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade methanol, HPLC grade chloroform and ACS grade formic acid (EMD Chemicals) were purchased from VWR (Bridgeport, NJ, USA). Cellular Proliferation Reagent WST-1 was purchased from Roche (Indianapolis, IN, USA).

### **2.2 HPLC ESI-MS/MS Conditions**

LPA subspecies were separated using a Kinetex 2.6u C18 100Å 50 x 2.1 mm reverse phase column on a Shimadzu 20-AD Series HPLC and subjected to mass spectrometric analysis using a ABSCIEX 4000 QTRAP. Mass spectrometry parameters were as follows: Polarity-Negative, Ion Source: Electrospray, Q1 Resolution: Low, Q3 Resolution: Unit, Collision Activated Dissociation: High, MCA: No, Curtain Gas: 15.0 psi, Ion Source Temperature:

400.0°C, Nebulizer Gas: 30.0 psi, Turbo Gas: 70.0 psi, Interface Heater: On, Ion Spray Voltage - 4500.0 V, Collision Cell Exit Potential: -9.0. MRM transitions with corresponding declustering potentials, collision energies and collision exit potentials are listed in Table 3.

HPLC conditions were as follows: Total Flow: 300 µl/min, Injection Volume: 10 µl, Column Oven: 50.0°C. Solvents for reverse phase HPLC separation were: Solvent A, 58:41:1 methanol:water:formic acid and solvent B, 99:1 methanol:formic acid. Both solvents contained 5 mM ammonium formate. Solvent conditions for HPLC separations were: 100% Solvent A from 0-1 minute, linear increase in minutes 1-7 from 100% Solvent A to 100 % Solvent B, 100% Solvent B from minutes 7-8, immediate switch from 100% Solvent B to 100% Solvent A at minute 8, 100% A for minutes 8-10.

### **2.3 1% FBS Experiments**

SKOV3-Zeo, SKOV3-ATX, DOV13-Zeo, and DOV13-ATX cells were plated in 10 cm dishes and grown to 80% confluency in 10% FBS supplemented RPMI. Cells were transferred to 1% FBS supplemented media overnight. Media was aliquoted into glass screw-top vials and stored at -20°C until HPLC ESI-MS/MS analysis and biological assays could be performed. Cells were harvested in 200 µl cold PBS. Results for HPLC ESI-MS/MS analysis were normalized to microgram protein from harvested cells using a Bradford assay.

### **2.4 LPA Extraction Procedures**

A modified Bligh-Dyer method was used to extract LPA from sample media. The extraction protocol was as follows: 100 µl of sample media, 1 ml chloroform, 500 µl methanol, 250 µl dH<sub>2</sub>O and 100 fmol 17:0 LPA standard were combined, vortexed, sonicated, and then

centrifuged at 4000 rpm for 10 minutes. The aqueous (top) layer was transferred to a clean glass tube, dried and resuspended in 100 µl methanol for mass spectrometry analysis and is represented as "Bligh-Dyer Aqueous". Organic (bottom) layer from the same extraction was transferred to a separate clean glass vial, dried and resuspended in 100 µl methanol for mass spectrometry analysis and is represented as "Bligh-Dyer Organic". After a separate Bligh-Dyer extraction was performed, the aqueous and organic layers were combined, dried and resuspended in 100 µl methanol for mass spectrometry analysis and are represented as "Bligh-Dyer"

A modified Folch method was also utilized for LPA extraction. The protocol was as follows: 200 µl sample + 2.5 ml chloroform + 1.25 ml methanol + 250 µl dH<sub>2</sub>O and 100 fmol 17:0 LPA standard were combined, vortexed, sonicated, and then centrifuged at 4000 rpm for 10 minutes. The aqueous (top) layer was transferred to a clean glass tube, dried and resuspended in 100 µl methanol for mass spectrometry analysis and is represented as "Folch Aqueous". Organic (bottom) layer from the same extraction was transferred to a separate clean glass vial, dried and resuspended in 100 µl methanol for mass spectrometry analysis and is represented as "Folch Organic". After a separate Folch extraction was performed, the aqueous and organic layers were combined, dried and resuspended in 100 µl methanol for mass spectrometry analysis and are represented as "Folch"

## **2.5 Cellular Migration Assay**

Cellular migration was measured in transwell chambers (pore size 8.0 µm; Corning Incorporated, Corning, NY) according to protocol in previous literature.<sup>40</sup> In short, transwells were coated with 0.1 mg/ml collagen and placed in lower chamber containing media from 1% FBS experiments detailed above. SKOV3-Zeo cells suspended in serum-free RPMI containing

0.1% fatty acid-free BSA were added to the upper chamber at  $2.5 \times 10^4$  cells/well. Cells were allowed to migrate for 6 hours under 5% CO<sub>2</sub> at 37°C. Top of insert filter surface was washed with PBS and then stained with 0.1% crystal violet in methanol for 10 minutes. Filter surface was washed three times with PBS, and non-migrated cells were removed from the top filter surface with a cotton swab. Migrated cells were stained by the crystal violet and counted under a microscope.

## **2.6 Cellular Proliferation Assay**

Cellular proliferation was measured using WST-1 based colorimetric assay. SKOV3-Zeo cells ( $5 \times 10^5$ ) were plated in five six-well plates and allowed to attach for 12 hours. 12 hours after plating, WST-1 reagent (200 µl) was added to four wells of one plate for reading of baseline attachment of SKOV3-Zeo cells. WST-1 reagent was allowed to incubate for 1 hour and absorbance was read at 450 nm with blank media absorbance used as background subtraction. After allowing cells to attach to the plate overnight, wells were washed once with PBS and 2 ml of conditioned media from the 1% FBS experiments was added to wells. Absorbance at 450 nm was measured using WST-1 reagent at 36 and 48 hours post-baseline reading. Cellular proliferation was calculated by subtracting the absorbance of blank media and average absorbance of 12 hour baseline measurement.



## Results

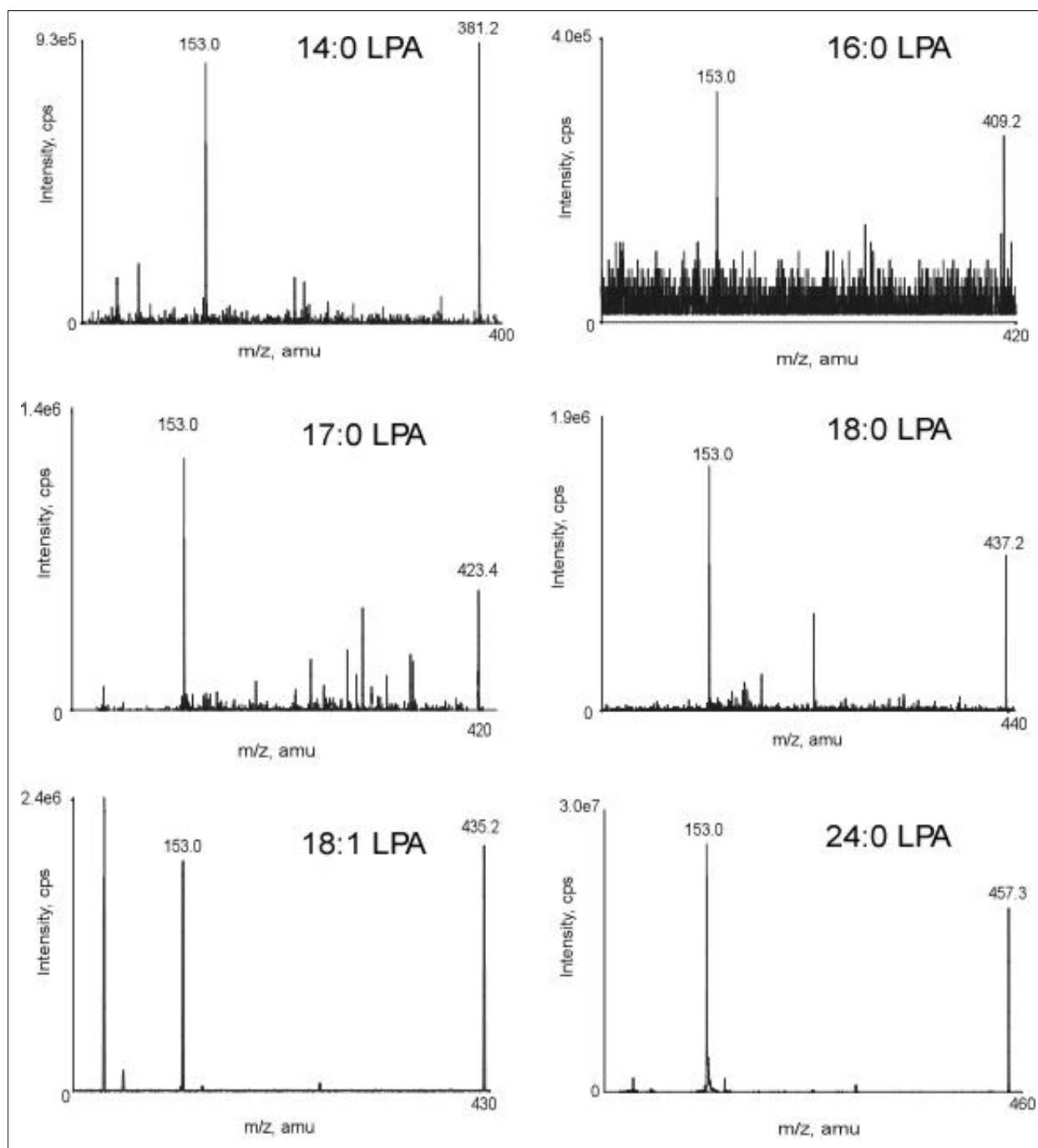
### 3.1 Retention Time Markers and Linear Signal Response Show Increased HPLC ESI-MS/MS Sensitivity for LPA Quantitation

Previous HPLC ESI-MS/MS methods have been limited in sensitivity allowing only for quantification of LPA from biological samples such as tissue, blood, plasma, or serum. The goal of this study was to produce an HPLC ESI-MS/MS method with a high degree of sensitivity to quantify LPA levels from cell culture. In this regard, distinct fragmentation patterns were required for determination of optimal mass spectrometer parameters for the detection of LPA subspecies (Figure 4). Quantitative optimization for the transition of precursor to product ions supplied the optimal ABSCIEX 4000 QTRAP parameters that are detailed in Table 3. There were three common product ions produced upon fragmentation of LPA subspecies: 79  $m/z$  - representing the fragmentation of the phosphite ( $\text{PO}_3^{-3}$ ) from the *sn*-3 position, 97  $m/z$  - representing the fragmentation of the phosphate ( $\text{PO}_4^{-4}$ ) from the *sn*-3 position, and 153  $m/z$  - representing the cyclical glycerol backbone produced upon fragmentation. The 153  $m/z$  product ion was chosen for method development as it is the product ion common to all subspecies that was produced with the highest intensity (Figure 4).

Reverse phase HPLC separation prior to ESI-MS/MS provided the ability to easily and accurately distinguish LPA subspecies in relation to specific retention times. Several HPLC solvent conditions were investigated for accurate separation. The traditional HPLC solvents for LPA analysis provided insufficient elution from the fused-core column and insufficient separation among subspecies (data not shown).<sup>37, 39, 41, 42</sup> In order to increase sample throughput, the HPLC solvent system used by the Chalfant laboratory for sphingolipid analysis was investigated for LPA analysis. Multiple variations of HPLC solvent gradients for this system

were attempted, but the best separation was provided by the solvent system and gradient detailed in Figure 5. Distinct chromatograms were produced (Figure 6) to allow accurate identification of LPA species related to specific HPLC retention times (Table 3). Peak assignments were initially determined by the analysis of indicated LPA standards using the MRM transitions and mass spectrometry parameters detailed in Table 3. Since there are no commercially available standards for 18:2 LPA and 22:6 LPA, relative retention times were calculated based upon the retention times for experimentally validated standards.

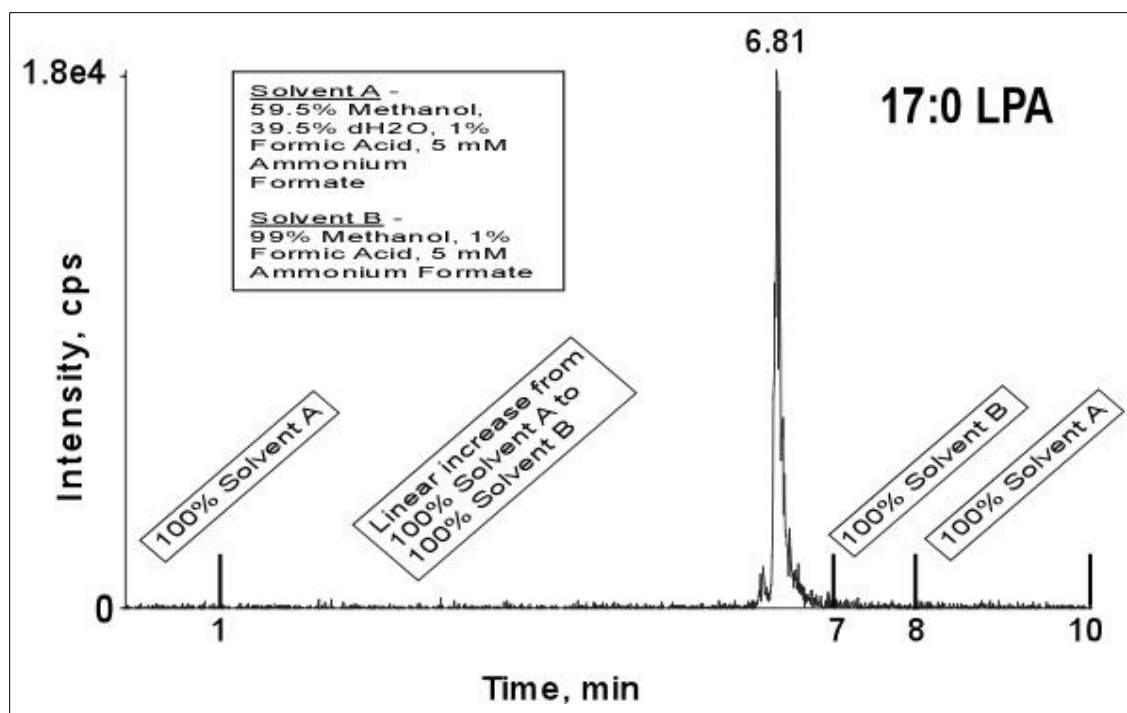
To investigate the sensitivity limits of this novel HPLC ESI-MS/MS method, a standard mixture of 14:0 LPA, 16:0 LPA, 17:0 LPA, 18:0 LPA, 18:1 LPA and 20:4 LPA was analyzed in concentrations from 10 fmols to 1 pmol per injection, and the signal response was measured as the area under the peak. When the results were plotted as signal response versus LPA concentration, a linear relationship was generated with correlation coefficients above 0.999 (Figure 7A). When the results were plotted as a log-log plot of signal response versus LPA concentration, a linear relationship was generated with correlation coefficients above 0.997 (Figure 7B). Intra-day and inter-day precision and accuracy of QC samples were within acceptable range (Table 4). The method produced a lower limit of quantitation (five times signal to noise ratio) of 10 fmol per injection for each chain length. This lower limit of quantitation represents a minimum of a five-fold increase in sensitivity from the most recent HPLC ESI-MS/MS LPA method and fifteen-fold increase in the remaining mass spectrometric reports in the literature for LPA analysis.<sup>37,43</sup>



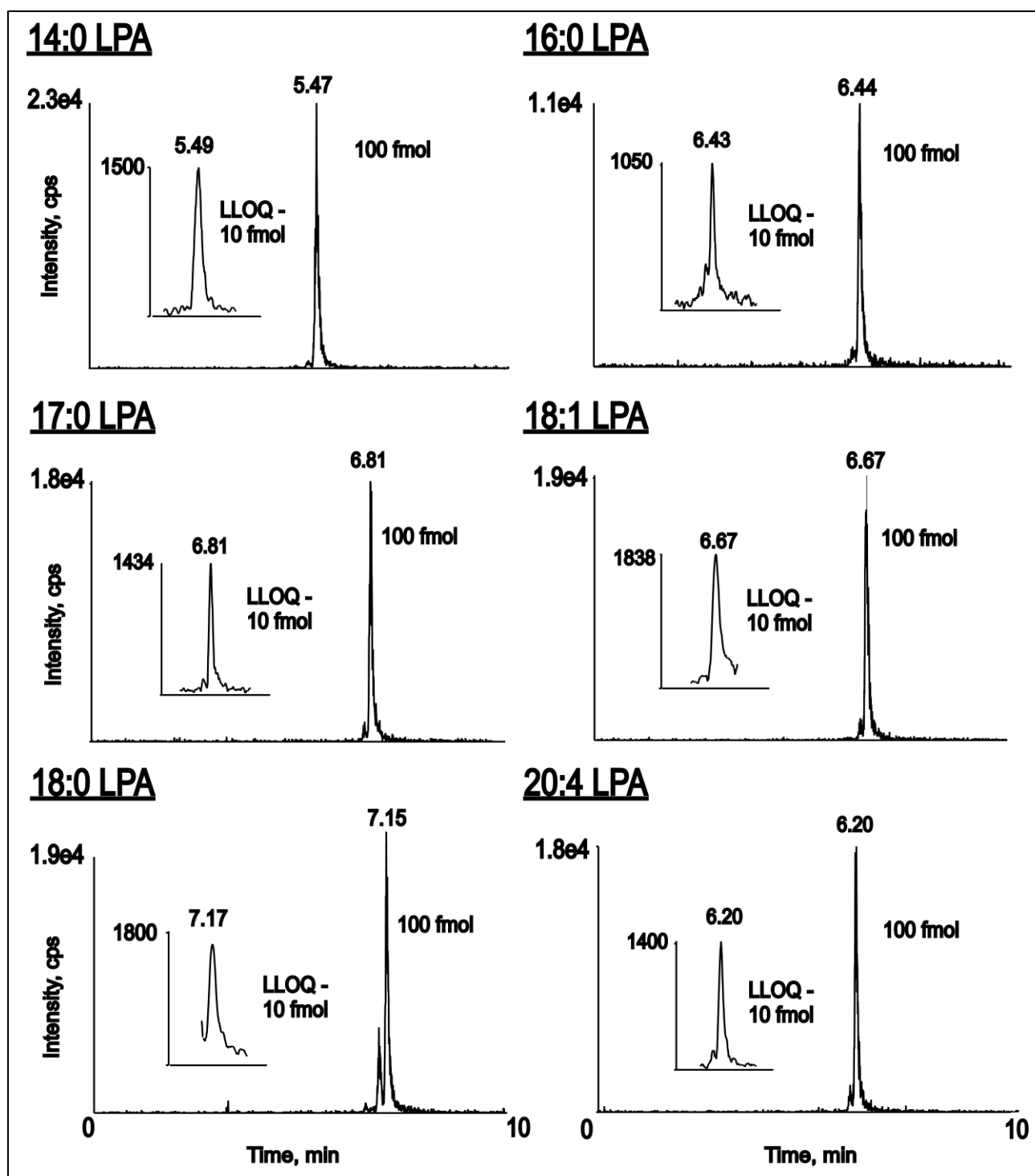
**Figure 4:** Fragmentation patterns are required for development of a mass spectrometric method for the analysis of LPA subspecies. Direct infusion of dilute standards into ABSCIEX 4000QTRAP provides distinct fragmentation patterns of precursor to product ions for each LPA subspecies. The 153.0  $m/z$  product ion represents the cyclical glycerol backbone produced by fragmentation of precursor LPA ions and is common to all LPA subspecies. The 153.0  $m/z$  product ion is therefore used during MRM analysis.

	Precursor Ion ( <i>m/z</i> )	Product Ion ( <i>m/z</i> )	DP	CE	EP	Retention Time (min)
14:0 LPA	381.3	152.7	-70.0	-26.0	-11.0	5.47
16:0 LPA	409.3	152.7	-60.0	-30.0	-12.0	6.44
17:0 LPA	423.3	152.7	-80.0	-30.0	-11.0	6.81
18:2 LPA	433.3	152.7	-80.0	-30.0	-10.0	6.14
18:1 LPA	435.3	152.7	-80.0	-30.0	-10.0	6.67
18:0 LPA	437.3	152.7	-60.0	-30.0	-12.0	7.15
20:4 LPA	457.3	152.7	-70.0	-30.0	-12.0	6.20
22:6 LPA	481.3	152.7	-70.0	-30.0	-12.0	6.14

**Table 3:** ABSCIEX 4000 QTRAP mass spectrometer settings and retention times for reverse phase chromatographic separation of LPA species as described in Materials and Methods. DP - declustering potential, CE - collision energy, EP - entrance potential.

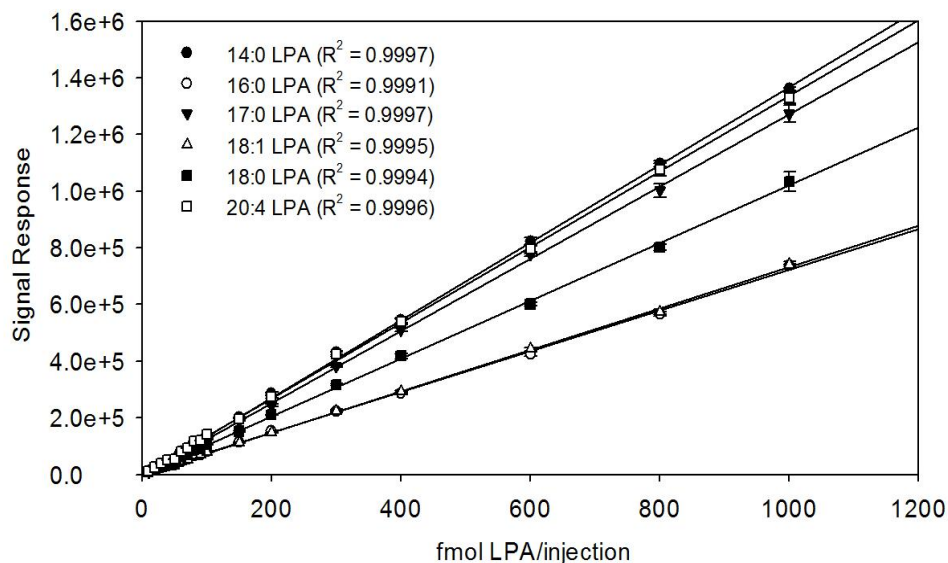


**Figure 5:** A novel solvent system allows for reverse phase HPLC separation of LPA subspecies at distinct retention times. A Kinetex 2.6u C18 100A 50 x 2.1 mm reverse phase column on a Shimadzu 20-AD Series HPLC provides optimal separation using the following HPLC parameters: Total Flow: 300  $\mu$ l/min, Injection Volume: 10  $\mu$ l, Column Oven: 50.0°C. Solvent conditions for reverse phase HPLC separation are: Solvent A, 58:41:1 methanol/water/formic acid and Solvent B, 99:1 methanol/formic acid. Both solvents will contain 5 mM ammonium formate. Solvent gradient for HPLC separation is: 100% Solvent A from 0-1 minute, linear increase in minutes 1-7 from 100% Solvent A to 100 % Solvent B, 100% Solvent B from minutes 7-8, immediate switch from 100% Solvent B to 100% Solvent A at minute 8, 100% A for minutes 8-10.

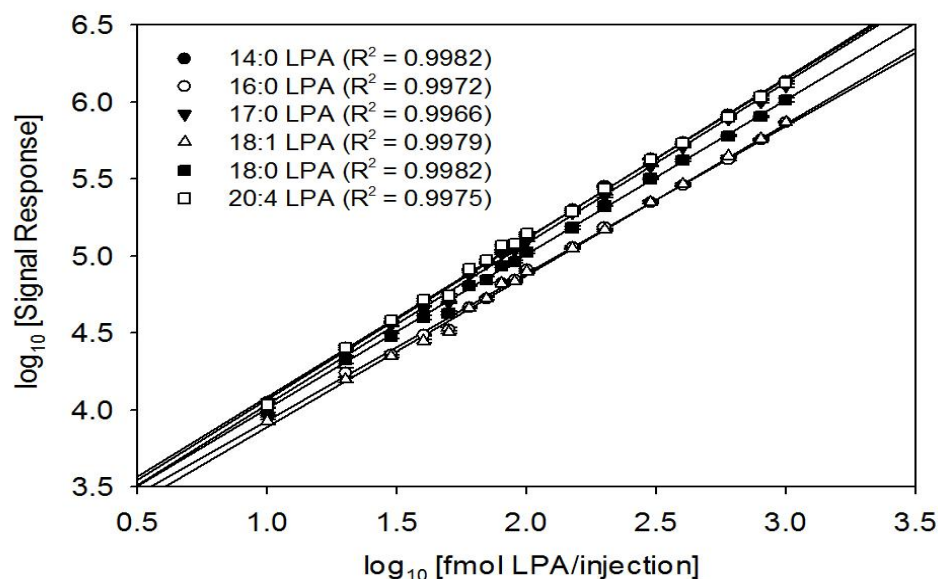


**Figure 6:** Retention time standards are required for unambiguous peak assignment in the quantitation of LPA species during reverse phase HPLC ESI-MS/MS analysis. An internal standard mixture of commercially available LPA standards produces distinct MRM chromatograms for 14:0 LPA, 16:0 LPA, 17:0 LPA, 18:1 LPA, 18:0 LPA and 20:4 LPA for the purpose of unambiguous peak assignment during HPLC ESI-MS/MS data analysis.

### Panel A



### Panel B



**Figure 7:** The method of detection for LPA species shows a linear response in the range from 10 fmol to 1 pmol for LPA standards. An internal standard mixture of 14:0 LPA, 16:0 LPA, 17:0 LPA, 18:0 LPA and 20:4 LPA was made in concentrations varying from 10 fmol/injection to 1 pmol/injection. (A) . The results were plotted as signal response (area under the peak) versus amount standard in fmol/injection. Data is the average of four separate sample injections  $\pm$  SEM. (B) The results were plotted as  $\log_{10}$  of signal response (area under the peak) versus  $\log_{10}$  fmol of standard per injection in a log-log plot. Data is the average of four separate sample injections  $\pm$  SEM.

LPA Species	Nominal Concentration (nM)	Intra-day precision and accuracy			Inter-day precision	
		Mean (n = 4) (nM)	Mean accuracy (%)	CV (%)	Mean (n = 4 days) (nM)	CV (%)
14:0 LPA	20	20.7	103.7	2.3	21.4	7.0
	40	41.8	104.5	6.8	42.1	10.0
	80	85.4	106.7	2.7	81.9	8.1
	100	101.8	101.8	10.3	98.4	11.7
16:0 LPA	20	21.4	107.2	3.3	20.5	8.8
	40	40.6	101.5	5.8	41.4	7.5
	80	75.1	93.8	4.9	76.0	7.2
	100	96.9	96.9	7.1	97.2	8.3
17:0 LPA	20	21.7	108.7	6.6	21.7	4.8
	40	42.3	105.9	4.1	42.4	6.1
	80	80.1	100.1	6.3	81.8	5.5
	100	103.4	103.4	3.2	101.0	6.6
18:1 LPA	20	20.7	103.3	1.9	20.6	9.3
	40	39.8	99.4	5.5	41.2	5.6
	80	76.3	95.4	3.8	82.0	6.9
	100	95.8	95.8	3.6	103.9	8.3
18:0 LPA	20	22.3	111.7	4.1	21.6	6.4
	40	43.5	108.7	3.5	42.1	5.6
	80	79.9	99.9	3.7	82.5	4.3
	100	100.3	100.3	3.9	100.2	4.7
20:4 LPA	20	21.4	107.2	10.3	20.0	11.7
	40	40.4	101.1	5.6	41.3	10.7
	80	75.2	94.1	6.0	83.2	10.6
	100	91.2	91.2	3.0	99.3	9.8

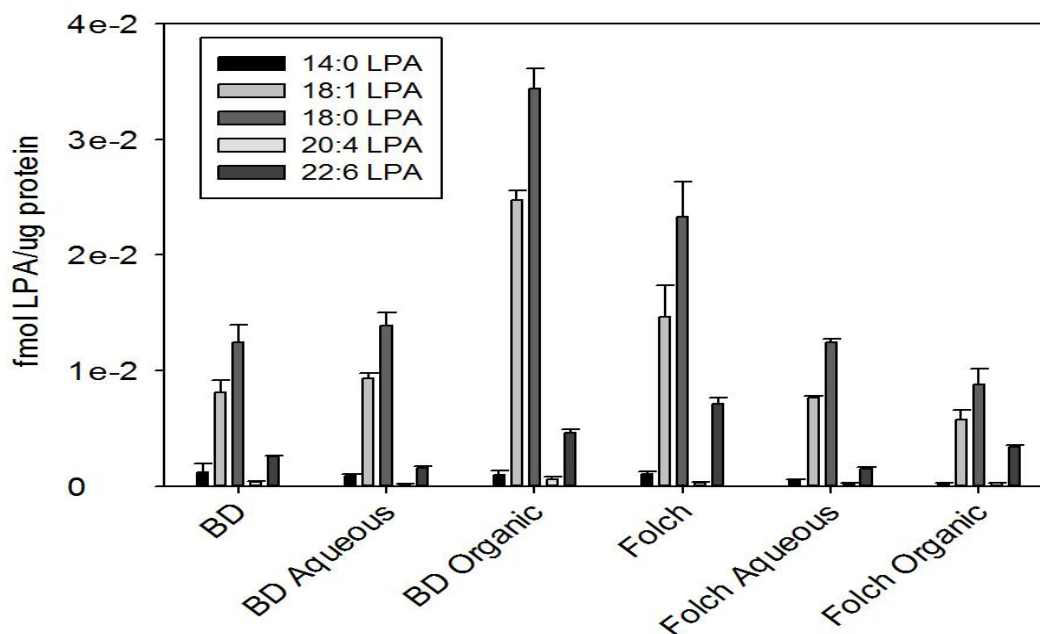
**Table 4:** Intra-day and Inter-day Precision and Accuracy



### 3.2 Non-acidified Extraction Methods Produce Quantifiable LPA Levels Without LPC Degradation

Most reported HPLC ESI-MS/MS methods for quantitation of LPA have relied on extraction methods that required highly acidic treatments. These acidic conditions may contribute to the artificial conversion of LPC to LPA, and thereby lead to LPA measurements that are not characteristic of actual sample levels.<sup>36, 39</sup> To address this issue, a number of extraction techniques without acidic treatment were tested on conditioned media samples. First, a direct media sample injection into the HPLC following polarity adjustment to starting HPLC conditions was tested. This extraction method proved to be insufficient due to a lack of sample concentration step during extraction (data not shown). Next, a simple lysophospholipid extraction that consists of a single methanol solvent dilution and single step of centrifugation was utilized as previously reported.<sup>44</sup> While this extraction was suitable for LPA extraction of *in vivo* samples, it was unsuccessful at providing sufficient recovery of the minute levels of LPA from *in vitro* samples because this protocol also lacked a sample concentration step (data not shown). A solid-phase extraction protocol utilizing a reversed phase silica based column was also tested but did not provide sufficient results due to an inability to efficiently remove LPA from the column during the final elution step (data not shown). Finally, two non-acidified liquid-liquid extractions termed modified Bligh-Dyer and modified Folch extractions were tested and showed to provide reliable recovery of LPA at levels above the limits of quantitation (Figure 8). Importantly, the Folch extraction technique demonstrated a more reliable recovery of LPA with the combined organic and aqueous fractions approximately equaling the total Folch recovery (Figure 8). Therefore, the total Folch extraction was used throughout the remainder of the manuscript due to

its consistent recovery, simple protocol, and lack of producing artificial LPA contamination under acidic conditions.



**Figure 8:** Non-acidic extraction methods for *in vitro* LPA quantitation show that organic-aqueous phase break protocols provided sufficient recovery for HPLC ESI-MS/MS analysis for 1% FBS overnight treated DOV13-Zeo cells. BD - combined layers of Bligh-Dyer, BD Aqueous - aqueous layer of Bligh-Dyer, BD Organic- organic layer of Bligh-Dyer, Folch - combined layers of Folch, Folch Aqueous - aqueous layer of Folch, Folch Organic - organic layer of Folch. Extraction protocols are detailed in Materials and Methods. Data represents  $n = 3 \pm \text{SEM}$ .

### 3.3 Autotaxin Overexpression Induces an Increase in Specific LPA Species

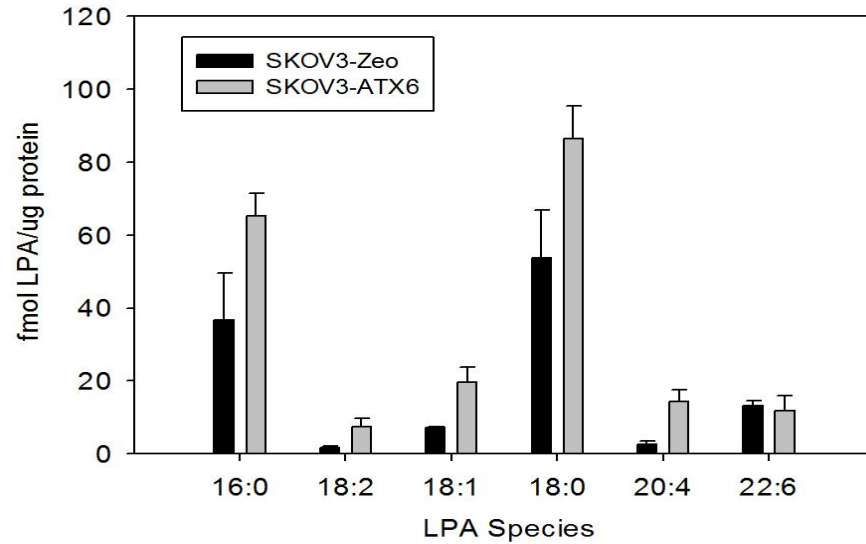
With the optimal extraction procedure for LPA determined, the protocol was fully operational for validation using biological applications. In this regard, we chose to examine the production of LPA in autotaxin-overexpressing cells as compared to vector control cells. Since ovarian cancer has been widely established to have increased LPA dysregulation, ovarian cancer cell lines SKOV3 and DOV13 and their corresponding autotaxin clones were analyzed. Figure 9A shows that the 16:0, 18:1, 18:2, 18:0, and 20:4 LPA subspecies were significantly increased in the media from SKOV3-ATX cells, but importantly, the 22:6 LPA subspecies remained relatively unchanged. Similarly, comparison of LPA subspecies in media from DOV13-Zeo and DOV13-ATX showed an increase in the 16:0, 18:1, and 18:0 LPA subspecies with little or no change among the 14:0, 20:4, and 22:6 LPA subspecies (Figure 10A). Hence, autotaxin increases the levels of specific chain lengths of LPA. Furthermore, the total levels of LPA increased two-fold in media from autotaxin-overexpressing cells in both cell lines studied, as indicated in Figures 9B and 10B.

Prior to the development of this HPLC ESI-MS/MS method for quantitation of LPA from cell culture, quantitation of LPA production have relied on comparisons to the effect of exogenously introduced LPA on biological assays such as cellular migration and proliferation.<sup>40,45</sup> Therefore, the observed increases in LPA levels produced by autotaxin overexpression as assayed by HPLC ESI-MS/MS were compared to the biological effect of these increased levels of LPA cellular migration and proliferation. Specifically, media from either SKOV3-Zeo or SKOV3-ATX was used as a chemoattractant in cellular migration assays. Media from SKOV3-ATX produced a two-fold increase in cellular migration when compared to media from SKOV3-Zeo, which correlated to the increase in LPA levels measured by HPLC ESI-

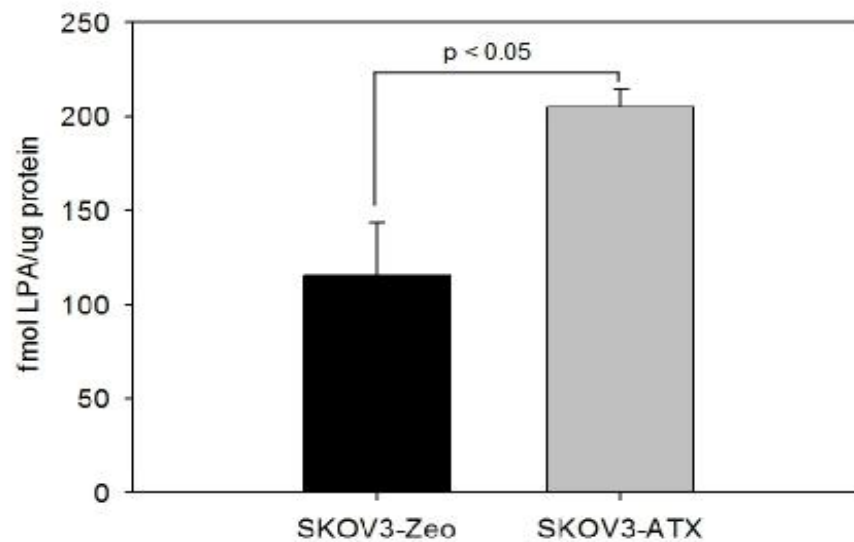
MS/MS (Figure 11). To further validate the increase in LPA levels in SKOV3-ATX media, the effect of SKOV3-ATX and SKOV3-Zeo media on cellular proliferation was measured. Media from autotaxin overexpressing cells induced an increased in cellular proliferation as compared to SKOV3-Zeo media, which also correlated to the total increase in LPA levels measured by HPLC ESI-MS/MS (Figure 12).

Migration and proliferation studies were also done with conditioned media from DOV13-Zeo and DOV13-ATX cells to validate the HPLC ESI-MS/MS analysis detailed in Figure 10. The approximate two-fold increase of cellular migration of SKOV3-Zeo cells using conditioned media from DOV13-Zeo and DOV13-ATX as chemoattractants correlated to the two-fold increase of total LPA levels as determined by HPLC ESI-MS/MS analysis (Figure 13). However, the SKOV3-Zeo cellular proliferation assays using conditioned media from DOV13-Zeo and DOV13-ATX showed no increase in cellular proliferation (Figure 14). This result shows that the HPLC ESI-MS/MS method developed in this study is superior to biological assays currently in use.

### Panel A

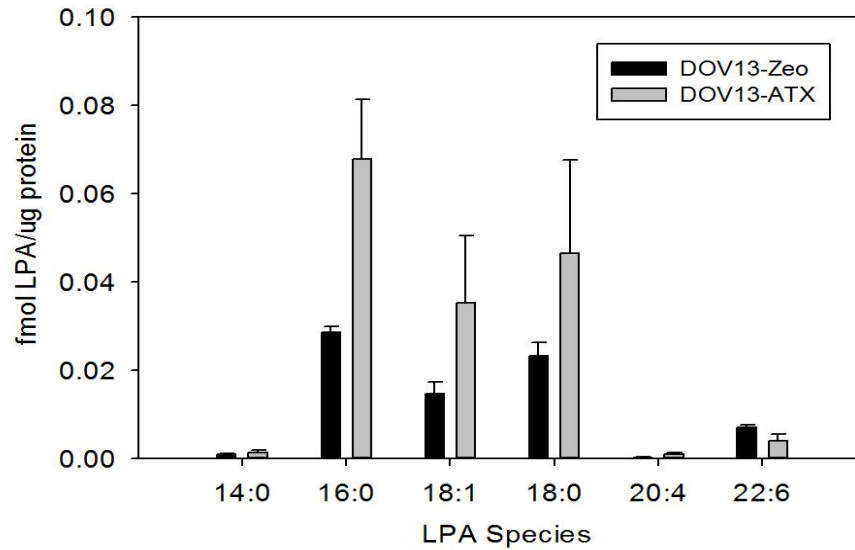


### Panel B

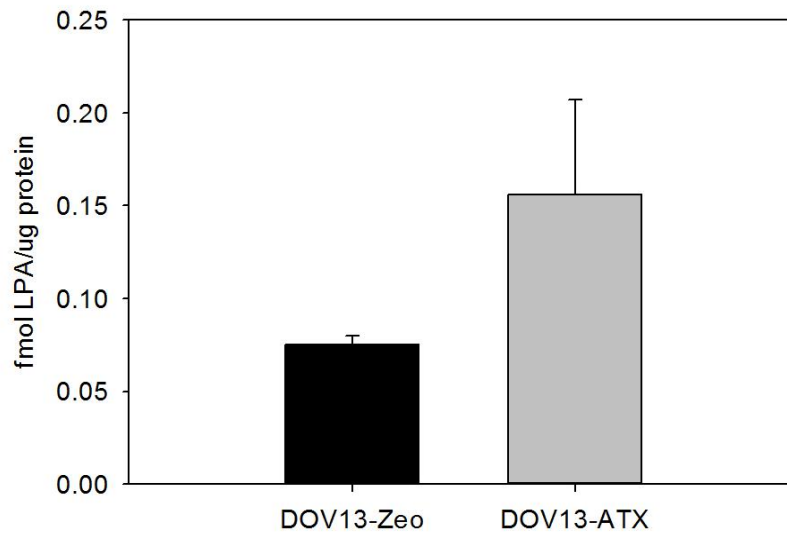


**Figure 9:** Comparison of control vector SKOV3 cells versus autotaxin-overexpressing clones show increased levels of LPA in autotaxin-overexpressing cell lines. (A) HPLC ESI-MS/MS analysis of LPA subspecies shows increases in 16:0, 18:2, 18:1, 18:0, and 20:4 LPA levels, but not 22:6 LPA. Data represents  $n = 3 \pm \text{SEM}$ . (B) HPLC ESI-MS/MS analysis detailed in (A) shows an approximate two-fold increase in total LPA levels between control vector and autotaxin-overexpressing cells. Data represents  $n = 3 \pm \text{SEM}$ . p-values were calculated as student's t-test using SigmaPlot v. 12.0 (SyStat).

### Panel A

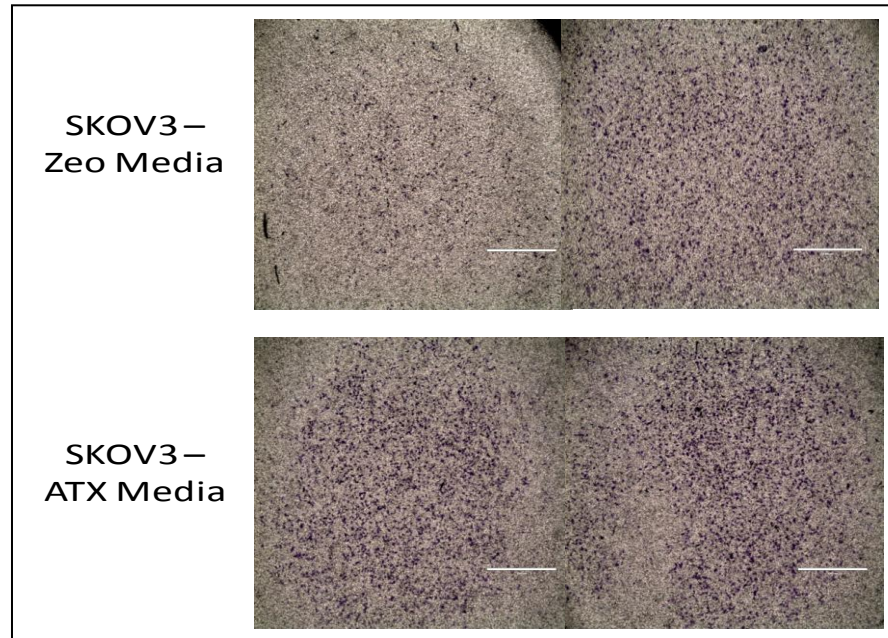


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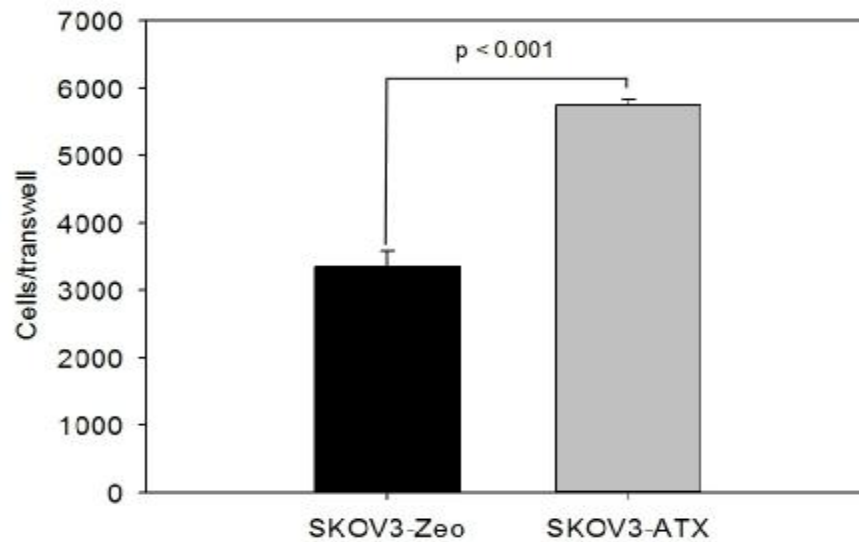


**Figure 10:** Comparison of control vector DOV13 cells versus autotaxin-overexpressing clones show increased levels of LPA in autotaxin-overexpressing cell lines. (A) HPLC ESI-MS/MS analysis of LPA subspecies shows increases in 16:0, 18:1, 18:0, and 20:4 LPA levels, but not 14:0 or 22:6 LPA. Data represents  $n = 3 \pm \text{SEM}$ . (B) HPLC ESI-MS/MS analysis detailed in (A) shows an approximate two-fold increase in total LPA levels between control vector and autotaxin-overexpressing cells. Data represents  $n = 3 \pm \text{SEM}$ .

### Panel A



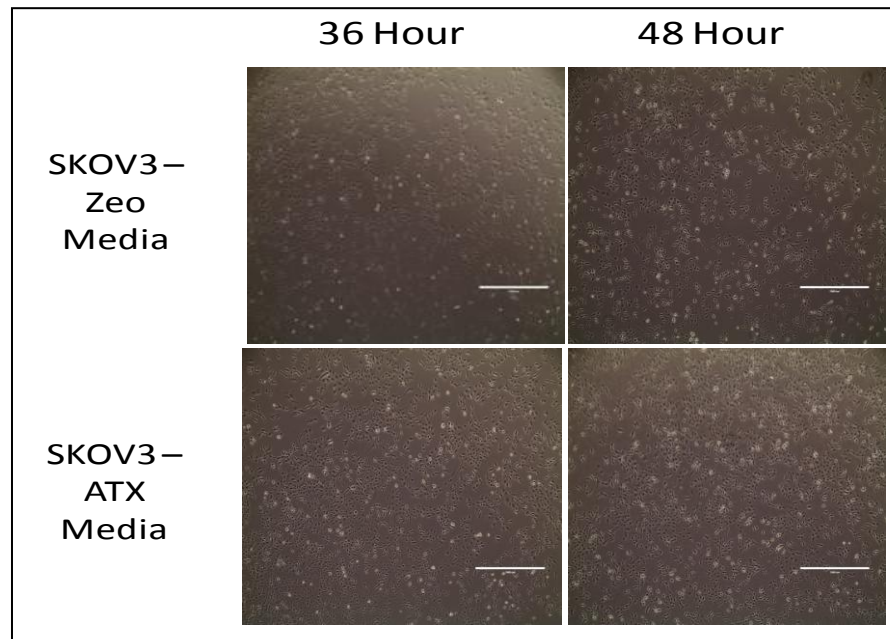
### Panel B



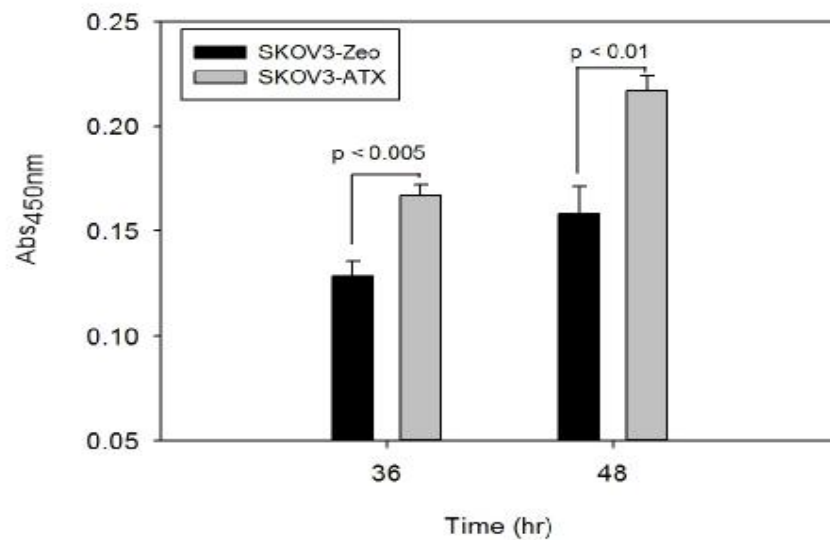
**Figure 11:** Conditioned media from SKOV3 autotaxin-overexpressing clones correspond to increased cellular migration of SKOV3-Zeo cells when compared to conditioned media from control vector cell lines. Media from overnight 1% FBS experimental protocol was used as a chemoattractant for cellular migration assay as detailed in Materials and Methods. (A) Images of migrated cells show increased SKOV-Zeo cellular migration using conditioned media from autotaxin-overexpressing cell line. (B) Calculations show that increases in migration correspond to total LPA levels measured by HPLC ESI-MS/MS analysis. Data represents  $n = 4 \pm \text{SEM}$ . p-values were calculated as student's t-test using SigmaPlot v. 12.0 (SyStat).



**Panel A**

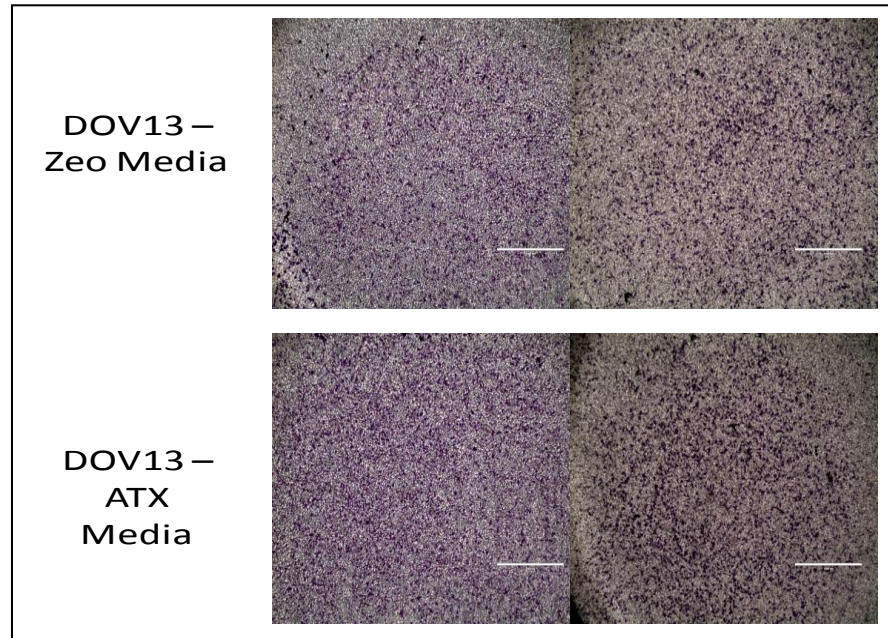


**Panel B**

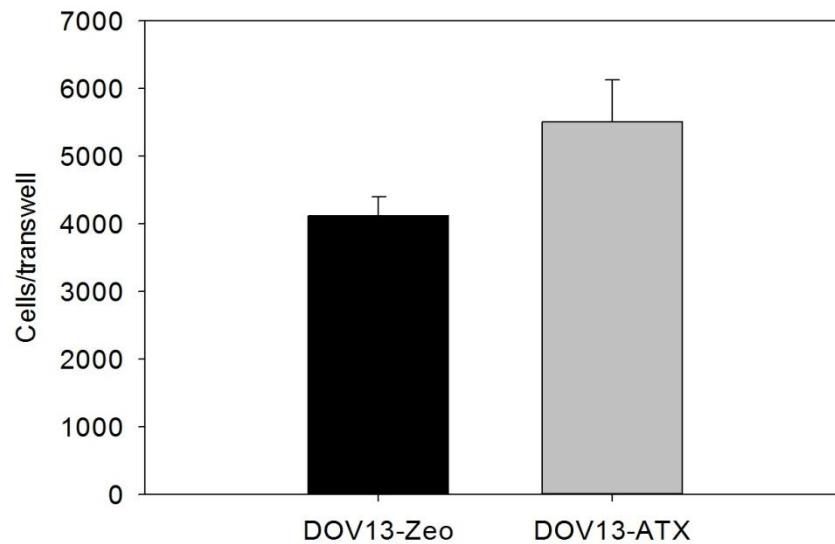


**Figure 12:** Conditioned media from SKOV3 autotaxin-overexpressing clones is associated with increased SKOV3-Zeo cellular proliferation when compared to conditioned media from control vector cell line. (A) Images of cell growth at corresponding time points show increased SKOV-Zeo cellular proliferation. (B) Calculations show that increases in proliferation correspond to total LPA levels measured by HPLC ESI-MS/MS analysis. Data represents  $n = 5 \pm \text{SEM}$ . p-values were calculated as student's t-test using SigmaPlot v. 12.0 (SyStat).

**Panel A**

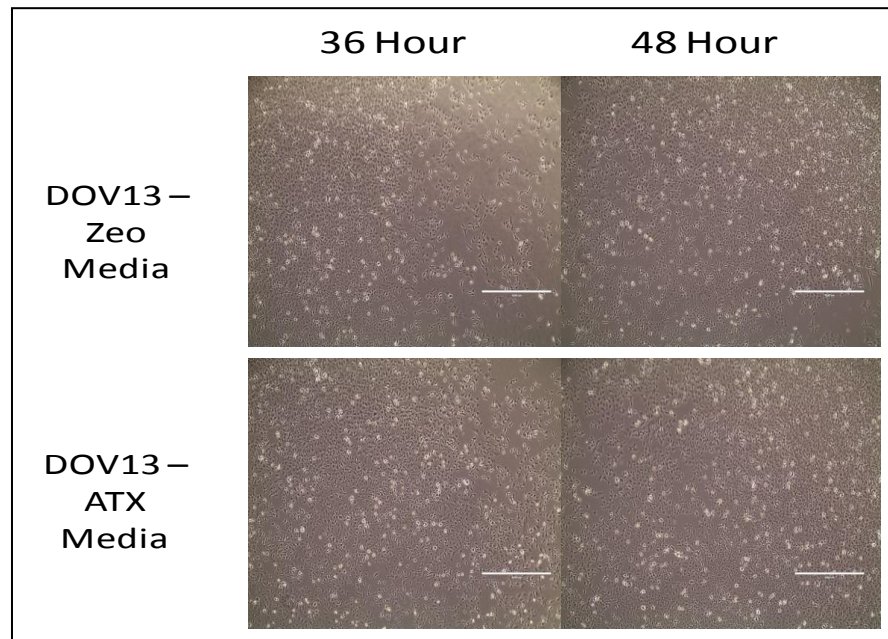


**Panel B**

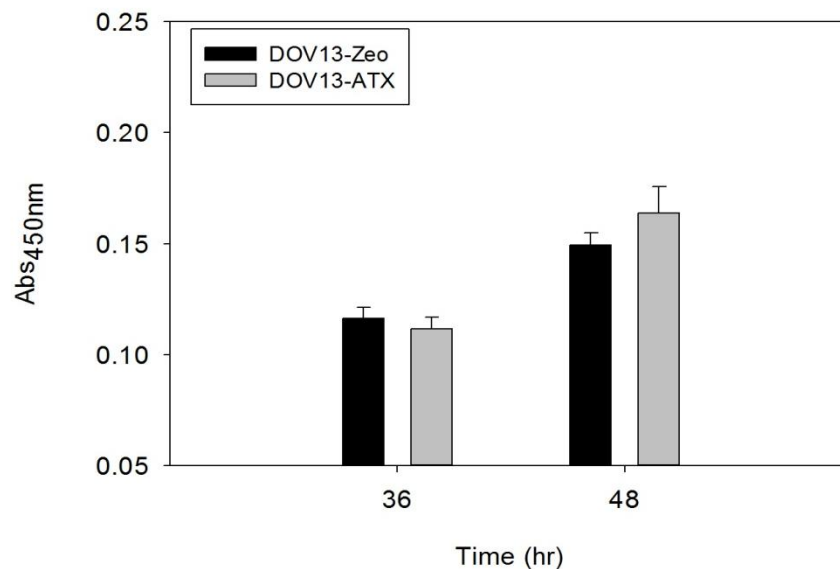


**Figure 13:** Conditioned media from DOV13 autotaxin-overexpressing clones correspond to increased cellular migration of SKOV3-Zeo cells when compared to conditioned media from control vector cell lines. (A) Images of migrated cells show increased SKOV-Zeo cellular migration using conditioned media from autotaxin-overexpressing cell line. (B) Calculations show that increases in migration mimic the increased total LPA levels measured by HPLC ESI-MS/MS analysis. Data represents  $n = 4 \pm \text{SEM}$ . p-values were calculated as student's t-test using SigmaPlot v. 12.0 (SyStat).

### Panel A



### Panel B



**Figure 14:** Conditioned media from DOV13 autotaxin-overexpressing clones does not correspond to increased SKOV3-Zeo cellular proliferation when compared to conditioned media from control vector cell line. (A) Images of cell growth at corresponding time points show no increase in SKOV-Zeo cellular proliferation. (B) Calculations show that, unlike SKOV3-ATX conditioned media, DOV13-ATX conditioned media does not cause an increase in SKOV13-Zeo cellular proliferation. Data represents  $n = 5 \pm \text{SEM}$ . p-values were calculated as student's t-test using SigmaPlot v. 12.0 (SyStat).

## Discussion

### 4.1 Necessity for this Novel HPLC ESI-MS/MS Method

The few reported methods for measuring endogenous LPA *in vitro* have suffered from a multitude of problems, such as inability to distinguish LPA species, a labor intensive nature, and high limits of detection. For example, some previous studies have even relied on thin-layer chromatography for pre-separation, a highly labor intensive and mainly qualitative protocol, for measuring autotaxin activity and subsequent LPA production *in vitro*.<sup>38</sup> Recently, HPLC ESI-MS/MS has been established as a reliable method for LPA quantitation from biological samples, but the significant limitations of these methods have made *in vitro* analysis unfeasible. In this study, we have developed a HPLC ESI-MS/MS method with increased sensitivity to resolve these issues and provide researchers the ability to make *in vitro* LPA quantitation possible in conjunction with quantitative measurements of other lipid classes.

### 4.2 Advantages of HPLC Conditions Utilized in this Study

Prior to this study, many of the publications utilizing HPLC ESI-MS/MS method development for LPA measurement suffer from undesirable HPLC conditions. For example, one report demonstrated what was termed "unidentified peaks" during chromatographic separation.<sup>37</sup> The HPLC conditions reported here allow for a more reliable identification of LPA species by the elimination of these unidentifiable peaks. This HPLC method also does not utilize undesirable solvent conditions. For example, the highly acidic solvents used by some methods may result in on-column degradation of LPA and artificial conversion of LPC to LPA.<sup>35</sup> Also, the highly organic solvents reported by other groups can affect the lifetime of the HPLC seals and are not functional for analysis of other lipid classes in the same sample run.<sup>36,39</sup> Use of such lipid

class specific HPLC methods significantly reduces the amount of data acquired from a single sample. The solvent system used in this study has previously been established for the study of sphingolipids, particularly ceramide, ceramide-1-phosphate, sphingosine, and sphingosine-1-phosphate.<sup>46</sup> Other HPLC solvent systems reported for LPA, including the most recent HPLC ESI-MS/MS method with highest sensitivity until this report, have the potential for ion suppression due to the inclusion of triethyl ammonium acetate as a modifier.<sup>43</sup> This suppression will produce the requirement of regular and thorough cleaning of the instrument before further analysis of other lipid classes. The need to change solvent systems to analyze the different lipid species also significantly increases valuable instrument time, thereby decreasing sample throughput. The HPLC ESI-MS/MS method in this study also utilizes solvent conditions that do not cause contamination and are capable of being used for sphingolipid analysis. Hence, this new HPLC ESI-MS/MS method for LPA not only overcomes the undesirable HPLC conditions but also uses chromatographic separation that allows for other lipids to be efficiently analyzed during the same sample run.

#### **4.3 Advantages of LPA Extraction Method Utilized in this Study**

An easy and reliable extraction method for LPA analysis was determined. Prior to this study, numerous extraction protocols have been published for the separation of LPA from biological samples, but early methods used thin-layer chromatographic separation as the initial basis of separation, and subsequent protocols have utilized a modified form of Bligh-Dyer lipid extraction where the optimal recovery is obtained using highly acidic conditions.<sup>37,38</sup> These acidic conditions have been shown to cause conversion of LPC to LPA during extraction and thereby provide inaccurate and artificial measurements.<sup>36,47</sup> Water-saturated butanol liquid-liquid

extraction methods have proven to be efficient, but are quite labor intensive.<sup>39,47</sup> The extraction method utilized in this study is a simple, efficient, organic-aqueous phase break separation that requires minimal labor, minimizes artificial LPA measurements, and provides sufficient recovery for LPA quantitation.

#### **4.4 Increased Sensitivity Provides the Ability for Quantitation of *in vitro* LPA Levels**

The method described herein provides a highly sensitive assay for the quantification of LPA levels from *in vitro* experiments. An increase in HPLC ESI-MS/MS sensitivity was necessary to quantify LPA subspecies levels from *in vitro* samples as other *in vitro* LPA quantitation methods had significant sensitivity limitations. The most sensitive HPLC ESI-MS/MS method previously reported for LPA subspecies quantitation has a lower limit of quantitation of 54 femtomoles on-column.<sup>43</sup> The method described in this study achieves a lower limit of quantitation of 10 femtomoles on-column. Hence, this represents a five-fold increase in sensitivity levels for HPLC ESI-MS/MS analysis. Furthermore, other reported studies could only assay one subspecies of LPA even when attempting to artificially induce LPA production by LPC treatment.<sup>48</sup> This reported method also suffered from the same acidic extraction conditions as previously published protocols, limiting even conclusions for this one LPA subspecies.<sup>48</sup> The increased sensitivity of the HPLC ESI-MS/MS method developed in this study will provide researchers with the ability to easily and accurately quantify all LPA subspecies levels *in vitro*. Furthermore, the method described in this study was the first to be validated by traditional biological assays.

#### **4.5 Conclusions and Future Directions**

Measurement of LPA *in vitro* has proven to be unreliable until the development of the HPLC ESI-MS/MS method described herein. Previous HPLC ESI-MS/MS methods have focused on biological samples, which contain high levels of LPA, and which have also suffered from unfavorable HPLC conditions, hazardous and inaccurate extraction protocols, and inadequate sensitivity levels. This new HPLC ESI-MS/MS method will offer researchers a valuable tool for determination of the activities of LPA subspecies for the purpose of delineating biological mechanisms and pre-clinical drug development for diseases such as diabetes, atherosclerosis, and cancer. The increased sensitivity levels will also provide clinicians with better ability to quantitate LPA with the goal of early detection of disease states. Overall, the HPLC ESI-MS/MS method presented in this study supplies a new tool for further exploration of the biological functions of LPA.

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## **Vita**

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