

Faculty of Biological and Environmental Sciences  
University of Helsinki  
*Academic Dissertation*

# Methods and tools for mass spectrometric lipidome analysis

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*To be presented for public examination with the permission of the Faculty of Biological and Environmental Sciences of the University of Helsinki, in Biomedicum Helsinki 1 (Haartmaninkatu 8), Seminar Room 3 on June 17<sup>th</sup>, 2011, at 12 noon.*

Helsinki 2011

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ISBN 978-952-92-9018-5 (paperback)  
ISBN 978-952-10-6969-7 (PDF)  
PDF version published at [ethesis.helsinki.fi](http://ethesis.helsinki.fi)  
Helsinki University Print  
Helsinki 2011

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# List of original publications

1. Koivusalo M, Haimi P, Heikinheimo L, Kostainen R, Somerharju P (2001) Quantitative determination of phospholipid compositions by ESI-MS: Effects of acyl chain length, unsaturation, and lipid concentration on instrument response. *J. Lipid Res.* 42:663-72.

Perttu Haimi and Mirka Koivusalo each contributed equally to the experiments described in this article.

2. Rockwood AL, Haimi P; Efficient Calculation of Accurate Masses of Isotopic Peaks (2006) *J. Am. Soc. Mass Spectrom.* 17:415-9

The algorithm was proposed by Alan Rockwood. The manuscript of the article was written jointly by Alan Rockwood and Perttu Haimi. Perttu Haimi implemented and validated the two programs (emass and qmass) described in the article and performed the accuracy and running time comparisons.

3. Haimi P, Uphoff A, Hermansson M, Somerharju P (2006) New software tools for analysis of mass-spectrometric lipidome data. *Journal of Analytical Chemistry.* 78:8324-31.x

The LIMSA program described in the article was designed and implemented by Perttu Haimi. Andreas Uphoff designed and implemented the SECD program. Perttu Haimi, Andreas Uphoff and Martin Hermansson all participated in testing the programs and writing of the manuscript.

4. Haimi P, Hermansson M, Batchu KC, Virtanen JA, Somerharju P (2010) Substrate efflux propensity plays a key role in the specificity of secretory A-type phospholipases. *J. Biol. Chem.* 285:751-60

Perttu Haimi, Krishna Chaithanaya Batchu and Martin Hermansson all contributed equally to this work. Perttu Haimi developed programs for processing the extensive data collected from the experiments. Perttu Haimi, Krishna Chaithanaya Batchu and Martin Hermansson performed experiments and participated in writing of the manuscript.

# Abstract

This thesis consists of four parts. The first part addresses issues related to quantitative analysis of phospholipid compositions of cells and tissues by mass spectrometry. In particular, the effects of phospholipid structure and concentration were investigated. The second part describes a new computational method for calculation of the unit resolution isotopic distribution for any compound, given the isotope abundances of the elements it consists of. In the third part, software (LIMSA) for processing mass spectrometric data from complex lipid samples was developed and tested. The fourth part of the thesis makes use of the tools listed above and a novel mass-spectrometric approach was developed to determine the substrate specificities of three secretory phospholipases in unprecedented detail.

# List of Abbreviations

CDP	Cytidine Diphosphate
CL	Cardiolipin
DAG	Diacylglycerol
DI	Direct Infusion
ESI	Electrospray Ionization
HPLC	High Performance Liquid Chromatography
LBPA	Lysobisphosphatidic Acid
MALDI	Matrix-assisted Laser Desorption/Ionization
MAM	Mitochondria Attached Membrane
MS	Mass Spectrometry
MS <sup>n</sup>	Multistage MS
NL	Neutral Loss
PA	Phosphatidic Acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PLA <sub>2</sub>	Phospholipase A type 2
PS	Phosphatidylserine
SM	Sphingomyelin
TAG	Triacylglycerol
TOF	Time-of-flight

# Chapter 1

## Introduction

Lipids comprise a very diverse group of compounds [Fahy et al., 2005]. They have various functions, such as signal transduction and energy storage, but most importantly, lipids are able to form membranes. Membranes surround the cell and its organelles and restrict the diffusion of water-soluble ions, nucleic acids, proteins and other metabolites. Membranes protect the cell and its metabolism from the interference of the outside, and allow the cell organelles to have specialized functions.

Mass spectrometry has proven to be an excellent method for the analysis of lipid compositions, owing to its great resolving power and sensitivity [Brugger et al., 1997]. Mass spectrometry is also very suitable for studying metabolism because it can distinguish between stable isotope labelled compounds and naturally occurring compounds, and thus can be used to follow the isotope label during the course of metabolic reactions. For example, isotope labelled compounds and mass spectrometry have been used to study cholesterol absorption in man [Godin et al., 2004] and metabolism of phosphatidylcholine in the lung surfactant [Bernhard et al., 2004].

As the mass spectrometric methods of lipid analysis have developed, a new field, *lipidomics* has emerged [Wenk, 2005], [Orešič et al., 2008], [German et al., 2007]. Lipidomics allow detailed high throughput analysis of complex biological samples, thus giving the possibility to answer the many unresolved questions of the regulation of lipid metabolism at the cell or organism level. Lipidomics strives for comprehensiveness, sensitivity and accuracy of analysis. The large amount of data from lipidomics experiments necessitates the use of software for data processing and analysis [Katajamaa and Orešič, 2007], [Song et al., 2009].

This work consists of development of mass spectrometric methods for the analysis of phospholipid compositions of biological samples, development of lipidomic data processing software, and application of these methods for the study of substrate specificities of secreted phospholipases.



## Chapter 2

# Review of the literature

This review briefly introduces lipids and their properties. An overview of mass spectrometry and electrospray ionization is also provided, as much as it relates to mass spectrometry of lipids, especially phospholipids.

### 2.1 Lipid classes

Lipids are a diverse group of hydrophobic or amphipathic compounds. The theoretical total number of distinct lipid molecules has been estimated to be close to 200000 [Niemelä et al., 2009]. Lipids can be grouped to e.g. fatty acids, simple glycerolipids, glycerophospholipids, sphingolipids, sterols, prenol lipids and polyketides [Sud et al., 2007], [Vance and Vance, 1996].

Fatty acids are carboxylic acids of variable length (typically 14-24 carbons) containing a varying number of double bonds. The double bonds in a naturally occurring fatty acid are typically of the *cis*-configuration and, depending on the distance of the first double bond from the methyl end, fatty acids are divided to the omega-3, 6, and 9 series. Hydroxy fatty acids are intermediates of fatty acid catabolism and constituents of some sphingolipids. While mammalian cells contain the necessary enzymes for synthesis, elongation and unsaturation of their fatty acids, they cannot synthesize fatty acids of the omega-3 series [Vance and Vance, 1996]. Fatty acids are commonly described using the C:D notation, where C denotes the number of carbons in the chain, and D denotes the number of double bonds.

Arachidonic acid (20:4) is the precursor for eicosanoids, including prostaglandins, thromboxanes and leukotrienes, which are involved in inflammatory response and blood clotting. They act as local hormones that coordinate the local response for stimuli [Vance and Vance, 1996] and are also involved in cancer [Wang and DuBois, 2010]. Eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6) are omega-3 fatty acids and precursors of resolvins, docosatrienes and protectins [Serhan, 2005b]. Oxidized fatty acids of phospholipids seem to be involved in apoptosis and in

age-related diseases [Domingues et al., 2008], and thus there is an increasing interest in mass-spectrometric analysis of these compounds [Tyurin et al., 2008].

Simple glycerolipids are divided to mono-, di- and triacylglycerols and are important for the energy metabolism of the cell. Large amount of energy can be stored in triacylglycerols as they are osmotically inactive and their acyl chains consist of highly reduced carbon [Vance and Vance, 1996]. Diacylglycerols are also important intermediates of phospholipid biosynthesis [Vance and Vance, 1996].

Glycerophospholipids (GPLs) are the most abundant constituents of the cellular membranes, followed by sphingolipids and sterols (sphingomyelin and cholesterol in mammalian cells). GPLs are composed of a glycerol backbone, two fatty acids, a phosphate group and the polar head group (see Figure 2.1). Typically, the *sn1* position of the glycerol moiety is occupied by a saturated fatty acid and the *sn2* position by an unsaturated fatty acid [Lands, 2000]. The *sn1* can also contain an ether or vinyl ether -linked alkyl chain. The GPL *classes* differ in respect of the head group, which can be ethanolamine, choline, serine, inositol, glycerol etc.

Phospholipids have many important roles in cells, i.e. they form the backbone of all membranes, participate in signal transduction and anchor some proteins to membranes [van Meer et al., 2008]. Especially PI is important in signal transduction as it is the precursor of polyphosphoinositoles, which are cleaved to phosphorylated inositol and DAG which activate e.g. protein kinase C [Martin, 1998]. PS is found in the outer leaflet of the PM of apoptotic cells and may play a role in removal of such cells [Tyurina et al., 2004].

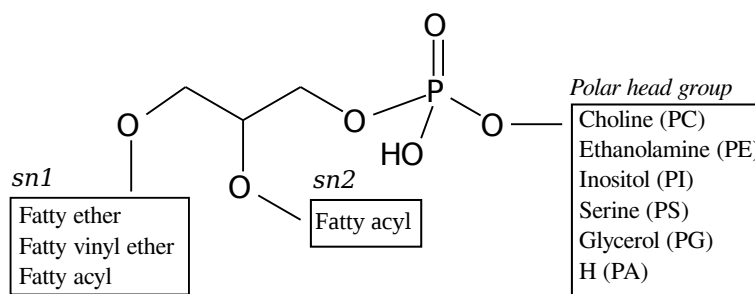


Figure 2.1: Phospholipid structure

Sphingolipids are composed of a sphingosine backbone, a fatty acid, and a polar “head group”. The head-group of sphingomyelin (SM), is phosphocholine, while the head groups of glycosphingolipids consist of one or more glycosyl moieties. Because of the diversity of these moieties, glycosphingolipids are structurally even more diverse than GPLs. Some sphingolipids

Lipid class	Mitochondria	ER	PM	lysosomes
PC	40.3	58.4	39.3	39.7
PE	34.6	21.8	23.3	14.1
PI	4.6	10.1	7.7	4.5
PS	0.7	2.9	9.0	1.7
SM	0.5	2.5	16.0	20.3
CL	17.8	1.1	1.0	1.0
LBPA	0.2			7.0

Table 2.1: Phospholipid compositions (%) of various organelles of rat liver [van Meer, 1989]

are rather hydrophilic (complex sphingolipids with many sialic acids, or sphingosine phosphate), while others can be very hydrophobic (ceramide) and thus no single extraction method provides good recovery of all sphingolipids [Merrill et al., 2005]. Sphingolipids, e.g. ceramides take part in signal transduction [Eyster, 2007], [Mills and Moolenaar, 2003]. Sphingomyelin and cholesterol have high affinity to each other [Bittman et al., 1994], and tend to form nanoscopic domains or *rafts* in cellular membranes [Simons and Gerl, 2010]. Glykosphingolipids have important roles in cell adhesion [Turner et al., 1992] and cellular recognition [Liberio and Mori, 2007].

## 2.2 Lipid compositions of cellular membranes

An eukaryotic cell contains many specialized organelles: The plasma membrane surrounding the cell, mitochondria, endoplasmic reticulum, the Golgi apparatus, lysosomes, etc. The lipid compositions of all these organelles differ, as shown in Table 2.1.

The lipid compositions of the cellular membranes are maintained by both metabolic and intracellular transport processes. The latter include vesicular trafficking, protein mediated and spontaneous diffusion as well as membrane contact site -dependent translocation (van Meer et al. [2008], Voelker [2005]). Regarding metabolism, cholesterol, ceramide and most phospholipids are synthesized in ER. PC is synthesized via the CDP-choline pathway in most cells but also via the PE methylation pathway in the liver [Houweling et al., 1995]. PE is synthesized via the CDP-ethanolamine pathway in the ER or by decarboxylation of PS in mitochondria [Vance and Vance, 2004]. PG and CL are both synthesized in the mitochondria [Schlame et al., 2000] and CL is only found in this organelle. PS is synthesized by a base-exchange reaction from PC (by PS synthase-1) or from PE (by PS synthase-2) in the ER and mitochondria-associated membranes (MAM). SM and glykosphingolipids are synthesized from ceramide in the Golgi apparatus. SM and PC are enriched

in the outer leaflet of the plasma membrane, while PE and PS are abundant in the inner leaflet [van Meer et al., 2008].

The maintenance of the lipid compositions of the various cellular membranes is poorly understood. Apart from synthesis and trafficking, phospholipases and acyltransferases play a significant role [Lands, 2000]. Phospholipases are classified according to the bond they hydrolyze. A -type phospholipases hydrolyze the ester bond between glycerol and a fatty acid [Schaloske and Dennis, 2006]. C and D -type phospholipases cleave between the phosphorous and the DAG moiety or between the phosphorous and the polar head group, respectively. Acyltransferases differ in their acyl specificity [Shindou and Shimizu, 2008], which may explain why different GPLs have different acyl chain compositions.

## 2.3 Lipid analysis

The progress in the analysis of lipid mixtures has been slower than for other biomolecules. One of the main reasons for this is the structural similarity of the molecular species belonging to same lipid class, which makes their separation difficult. Another reason is that the relative abundances of different lipids can vary several orders of magnitude which poses problems in their detection.

Earlier studies concentrated in the analysis of phospholipids at the class level with using thin-layer chromatography (TLC) or high performance liquid chromatography (HPLC) [Sderberg et al., 1991], [Patton et al., 1982]. Another “workhorse” in lipid analysis has been gas chromatography, which is used to analyze the fatty acid profiles of lipid classes, which had to be first separated using TLC or HPLC. Alternatively, the molecular species in a lipid class could be obtained by reverse-phase separation of molecular species of intact lipids. This approach, however, requires large amounts of lipids (hundreds of nanomoles) [Patton et al., 1982], thus making it unsuitable for e.g. the analysis of sub-cellular fractions from cultured cells. Also, complete separation of all molecular species is rarely achieved.

Mass spectrometry has opened up new possibilities for lipid analysis due to its high resolving power, sensitivity and the possibility to do structural identification by fragment analysis. Initially, the ionization methods available caused extensive fragmentation of the lipid molecules. This prevented detailed analysis of biological samples typically containing hundreds of lipid species. The introduction of *Electrospray* ionization (ESI), developed by Fenn and coworkers [Fenn et al., 1989] and the advances in instrumentation revolutionized the analysis of lipid compositions [Brugger et al., 1997]. ESI is a soft ionization method, i.e. it avoids unwanted fragmentation the lipids, and it is also compatible with on-line HPLC separation.

This novel method of analyzing lipid compositions lead to the introduc-

tion of the term *lipidomics* [Han and Gross, 2003]. Besides ESI, matrix-assisted laser desorption ionization (MALDI) has been used for lipidome analysis [Schiller et al., 2004]. MALDI is particularly useful for mapping of lipid distributions in tissues [Burnum et al., 2009].

## 2.4 Mass spectrometry of lipids

### 2.4.1 Principles of mass spectrometry

Mass spectrometry is based on the behavior of *gas phase ions* in an electric or magnetic field. The ions are accelerated in the field and the degree of acceleration depends on the charge and the mass of the ion. For this reason, mass spectrometers always measure the *mass-to-charge ratio* or  $m/z$  ratio of ions. There are various types of mass spectrometers but they all have following things in common: an ion source, a mass analyzer and a detector. The ion source generates ions of the analytes, the mass analyzer determines the mass-to-charge ratios of the ions generated and the detector counts the ions arriving from the mass analyzer [Gross, 2004]. Some instruments, like the Orbitrap [Hu et al., 2005] do not have a separate detector, because the mass analyzer is also used for detection.

Several types of mass analyzers with their particular advantages and disadvantages are employed in lipidomics [Milne et al., 2006] The mass analyzer and detector must be kept under high vacuum, which means that mass spectrometers are accompanied by vacuum pumps. Modern mass spectrometers are always accompanied by a computer which runs the control and data processing software.

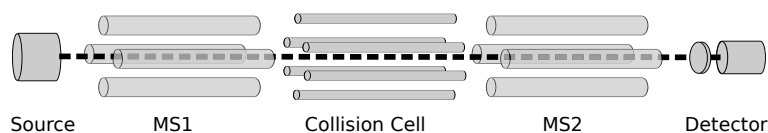


Figure 2.2: The components of a triple-quadrupole mass spectrometer. MS1 and MS2: the first and second mass analyzers. Collision cell can be used for fragment analysis by collision induced dissociation.

The most commonly used analyzer in lipidomics studies is the triple-quadrupole (Fig. 2.2). In such instruments, the ions entering the instrument first pass a mass analyzer (quadrupole 1), then a collision cell and finally the second mass analyzer (quadrupole 2). The fragments created in the collision cell (if active) provide direct information on the structure of the analytes. Common fragmentations are the loss of water, amine or methyl groups [Smith and Busch, 1999], but in regard to phospholipids, more useful information is provided by the fragmentations involving fatty acid and head

group moieties [Pulfer and Murphy, 2003].

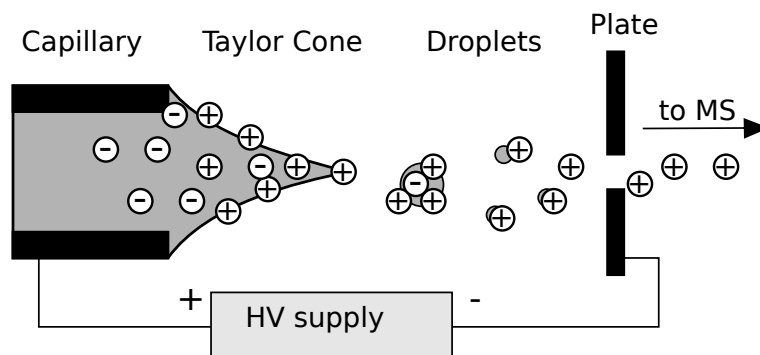


Figure 2.3: Electrospray ionization process [Cole, 1997]. The electric field created by the high voltage power supply drives the formation of the Taylor Cone at the tip of the spray needle. The high surface charge of the cone leads to ejection of small ESI droplets. Gas phase ions are formed when the solvent evaporates from the droplets. Ions enter the mass spectrometer for further analysis.

In electrospray ionization (Figure 2.3) the dissolved analytes are introduced to the source through a capillary, set to a high potential (voltage) relative to the entrance of the mass analyzer. The electric field drives the formation of the Taylor cone with highly charged surface at the tip of the capillary. The high charge density causes the formation of small droplets which are pulled towards the entrance of the instrument by the field. The droplets shrink as the solvent evaporates and eventually naked gas phase ions are formed [Cole, 1997], [Kerbale, 2000], [Cole, 2000], [Cech and Enke, 2001a].

Different ions partition between the surface and the interior of the charged solvent droplets, depending on their polarity and other properties. Salts or other easily ionizable impurities in the sample can cause pronounced loss of intensity of the analyte peaks. This is because salt ions tend to occupy the surface of the droplets, thus out-competing the analyte and reducing the efficiency of their ionization [Constantopoulos et al., 1999]. This *suppression* depends on the total concentration of the sample [Enke, 1997]. This phenomenon, also called the *matrix effect* complicate the quantification of the analyte, because the measured analyte ion intensity does not depend only on its own concentration, but is also affected by the amount of other ions present in the sample [Tang and Kebarle, 1993].

Interestingly, it has been shown that the ion intensity of an analyte correlates with its surface activity [Cech and Enke, 2001b], [Tang and Kebarle, 1993] and the retention time in a reverse-phase column [Cech et al., 2001]. Lipids are special among biomolecules as they form series of homologues

GPL class	Fragment	Precursor	Mode	polarity
PC	phosphocholine	[M+H] <sup>+</sup>	Par 184	positive
PE	phosphoethanolamine	[M+H] <sup>+</sup>	NL 141	positive
PS	serine	[M-H] <sup>-</sup>	NL 87	negative
PI	inositol phosphate	[M-H] <sup>-</sup>	Par 241	negative

Table 2.2: Specific scan modes for phospholipid classes (Par: parent scan, NL: neutral loss)

with systematically varying chain length or unsaturation. Thus, molecules with different surface activity and hydrophobicity exist within a lipid class. In ESI-MS, this property of lipids results in varying instrument response [Han and Cross, 1994]. For phospholipids, the instrument response has been shown to decrease with increasing acyl chain length [Brugger et al., 1997]. It has also been observed that the instrument response increases with the degree of unsaturation [Ishida et al., 2004], which is consistent with the fact that unsaturation decreases the hydrophobicity of the lipid molecules. In addition, the relative intensities can vary as function of the total phospholipid concentration [Zacarias et al., 2002], [Han et al., 2006], which further complicates their quantitative analysis.

While tuning of instrument settings, especially the collision energy in tandem MS, can be used to make the relative responses of the different lipid species more similar to each other, [Han, 2002], [Han and Gross, 2004], [Schwudke et al., 2006], this does not overcome the concentration and matrix effects, which can vary significantly between samples.

## 2.4.2 Fragmentation of phospholipids

Phospholipids contain a polar head group, the glycerol backbone and two fatty acids (Figure 2.1). Collision induced dissociation of phospholipids creates many informative fragments. Perhaps the most useful ones are those deriving from the head group (Table 2.2), since they allow one to do lipid class specific scans [Han and Gross, 2004], [Pulfer and Murphy, 2003]. Also, the fragmentations involving the fatty acid ester bond are useful as they provide information on the acyl chains either directly or indirectly based on the lyso-phospholipid fragments [Ekroos et al., 2003], [Pulfer and Murphy, 2003]. When using such specific scanning modes, one must be aware of overlapping (non-specific) fragmentations [Yan and Caldwell, 2004]. It is also notable that the intensities of the fragment ions can vary depending which adduct-forming ions (e.g.  $H^+$ ,  $Na^+$ ,  $Li^+$ ) are present [Han and Gross, 1995], [Hsu and Turk, 2003].

Positionisomers are difficult to identify using mass spectrometric methods because they usually produce the same fragment ions. However, the relative

abundances of the acyl chain loss ions often differ significantly and can be used for identification [Simes et al., 2008]. It has been observed that the ester bond of the sn2 fatty acid is more labile [Hsu and Turk, 2008b].

Identification of the double bond positions in the fatty acids of lipids relies on multistage MS ( $MS^n$ ) [Mitchell et al., 2009]. Polyunsaturated fatty acids are particularly difficult to identify, because of fewer informative product ions. Lithium adducts [Hsu and Turk, 2008a] and chemical derivatization by osmium tetroxide [Moe et al., 2005] or ozone [Thomas et al., 2007] have been successfully used to identify the double bond positions in phospholipids.

### 2.4.3 Instrumentation

The two main approaches used in mass spectrometric analysis of lipids are 1) direct infusion MS/MS and 2) LC-MS (liquid chromatography coupled to a mass-spectrometer). Although many kinds of mass spectrometers can be used for lipid analysis, triple-quadrupole instruments are the most commonly used ones because they allow one to carry out lipid class-specific MS/MS scans or allow sensitive selected reaction monitoring (SRM) in the context of LC-MS. Ion trap instruments are sensitive, and can be used for detailed structural analysis of lipids by using  $MS^3$  fragmentation [Ekroos et al., 2003].

Hybrid instruments which combine a time-of-flight (TOF) analyzer to quadrupole analyzer provide a better duty cycle and resolution than triple quadrupole instruments [Schwudke et al., 2006], [Taguchi et al., 2005]. These instruments can simultaneously perform multiple precursor ion scans, allowing e.g. both the head group and fatty acyl -specific scans to be performed at the same time [Zehethofer and Pinto, 2008]. By using special software, this enables the identification and quantification of a large number of lipids from a single analysis [Ejsing et al., 2006].

The LTQ Orbitrap instrument has a very high mass resolution and allowed one to resolve PS and PE species that differ by 0.0726 Da [Schwudke et al., 2007]. Koulman et al. [2009] were able to distinguish lyso PC18:3 from the sodium adduct of lyso PC16:0, the monoisotopic peaks of which differ by 0.0024 Da, using high resolution extracted ion chromatography.

Fourier transform ion cyclotron resonance mass spectrometry can have even higher resolution and has been used to identify e.g. the PE species in *C. elegans* and PC species in soybean [Ishida et al., 2004]. This instrument could distinguish the isotope peak of PC34:2 containing two  $^{13}C$  from the monoisotope peak of PC 34:1, which differ only by 0.009 Da.

Most mass spectrometers have a wide dynamic range, i.e. the measured intensity is proportional to the amount of ions in the sample. The range is limited by detector saturation and the charge space phenomenon at higher concentrations [Oursel et al., 2007]. The linear concentration range also



depends on the type of instrument and the rate the sample is introduced into the ion source. For example, a linear range of only up to  $2 \mu M$  has been reported for Micromass Quattro II triple quadrupole instrument with a conventional ESI source [DeLong et al., 2001], whereas upper limit of  $100 \mu M$  was reported for QSTAR pulsar i hybrid instrument equipped with a nanoflow ion source [Ejsing et al., 2006].

### **Direct infusion mass spectrometry**

Direct infusion mass spectrometry (DI-MS) with lipid class -specific scanning has been widely used in lipidomics [Brugger et al., 1997], [Hsu and Turk, 2009], [Yang et al., 2009b], [Gross and Han, 2009]. Direct infusion has the advantage over LC-MS that the electrospray conditions remain constant, i.e. the solvent composition, matrix and sample concentration, which can affect the ionization of analytes, do not change during the run.

The main disadvantage of DI-MS is that quantification of isobaric species in a lipid class is not possible using class-specific scanning but additional fragmentation analyses are required. Also, non-specific fragmentations and chemical background limit the dynamic range, thus hampering the quantification of the minor species [Yetukuri et al., 2008]. These problems can be partially mitigated with high resolution instruments.

Nanospray allows one to use low flow rates and thus can accommodate smaller samples. Also, nanospray seems to be less sensitive to suppression effects, presumably because the smaller size of the droplets allow a larger fraction of the ions to evaporate and enter the analyzer [Enke, 1997], [Schwudke et al., 2006]. Several groups have used a chip-based nanospray source (Advion NanoMate) in lipidomics [Yetukuri et al., 2008], [Ahn et al., 2007], [Ejsing et al., 2006].

### **Liquid chromatography - mass spectrometry**

A chromatographic step can be included before the mass analysis either online [Wang et al., 2005], [Sommer et al., 2006], off-line [Houjou et al., 2005] or both [Hutchins et al., 2008]. In a normal phase (e.g. silica or diol-modified silica) chromatography phospholipids are separated according the polar head group i.e. the different classes elute at different times [Hermansson et al., 2005]. Reverse phase chromatography employs hydrocarbon (typically C18) modified stationary phases and separate the lipids based on the hydrophobicity of their acyl chains. Separation of isobaric species can be achieved in some cases. In the analysis of sphingolipids, normal phase chromatography can be employed to separate the classes differing in the polar head group structure (e.g. glycosylceramides from lactosylceramides), while reverse phase chromatography allows separation of the molecular species in each class based on their different fatty acyl and/or sphingosyl residues

[Sullards et al., 2007]. LC-MS analysis is typically more sensitive than that using DI-MS because suppressing impurities are separated from the analytes.

On the other hand, the chromatographic step increases the time and cost of the analysis and creates problems like column memory or selective lipid losses in chromatography [DeLong et al., 2001]. Accurate quantification is also more difficult, because the composition of the eluent, which affects ionization efficiency, typically changes during the run.

#### 2.4.4 Sample preparation and introduction to MS instrument

Usually, the first step in lipid analysis is extraction with a mixture of organic solvents, typically chloroform and methanol. It is often not possible to optimally extract all lipids with one method and thus one has to combine two methods, such as normal Folch and acidic solvent extractions [Wakelam et al., 2007]. Ejsing et al. [2009] used 2-step extraction protocol to optimally recover both polar and apolar lipids from yeast cells. Solid phase extraction methods have also been used to extract lipids [Shen et al., 2005] [Mallet et al., 2004].

Phospholipids can hydrolyze during storage, especially if the solvent is basic [James et al., 2006]. Hydrolysis can be used to advantage. For instance, treatment with LiOMe hydrolyzes glycerophospholipids thus facilitating the analysis of SM [Yang et al., 2009b]. Polyunsaturated acyl chains are also prone to oxidation during storage and thus antioxidants are often added.

In DI-MS, the solvent composition is chosen so that it allows for optimal ionization of the analytes. A typical choice is a mixture of chloroform and methanol. In LC-MS, the properties of the column matrix dictate the choice of solvent.

Additives that improve ionization of lipids are often used in lipidomics. A commonly used additive is ammonia, which can be used both in positive and negative mode. In the positive mode, ammonia facilitates the “wrong-way-round” ionization [Mansoori et al., 1997], i.e. the observation of  $[M+H]^+$  ions in strongly basic solutions.

Addition of NaOH produces intense sodium adducts ( $[M+Na]^+$ ), while LiOH produces lithium adducts ( $[M+Li]^+$ ) with different fragmentation patterns ([Hsu et al., 1998], [Yang et al., 2009b], [Han and Gross, 2004]). In the negative mode, Cl ions [Han and Gross, 1995], ammonium acetate or formate [Mallet et al., 2004] or methyl amine [Ejsing et al., 2009] can be added to improve ionization or induce adduct formation. The additives which do not evaporate readily tend to contaminate the instrument.

## 2.5 Lipidomics

Lipidomics [Orešič et al., 2008], [German et al., 2007] [Merrill et al., 2005], [Wenk, 2005] [van Meer, 2005] is a branch of metabolomics [Robertson, 2005]. Initially, NMR was used in metabolomics [Bales et al., 1984], while mass spectrometry was implemented later [Plumb et al., 2002]. Lipidomics research could be roughly divided to four branches: 1) lipid fingerprinting, 2) biomarker discovery, 3) targeted analysis and 4) dynamic lipidomics. Lipidomics has mostly focused on the analysis of the lipid proper [Han and Gross, 2004], [He et al., 2007], [Merrill et al., 2005], but there is increasing interest to analyze also water soluble intermediates or break-down products (choline, phosphocholine, glycerophosphocholine, etc.), because that allows one to study lipid turnover as well.

Lipidomics has been applied to study a number of health-related issues, e.g. the cardiovascular disease, metabolic syndrome and diabetes [Gross and Han, 2007]. Han et al. [2005] studied the heart tissue of mice with streptozotocin induced diabetes. They found dramatic and progressive decrease of 18:2 fatty acid containing cardiolipin (CL) and increase of 22:6 fatty acid containing cardiolipin, thus indicating altered CL remodeling in the mitochondria. Lankinen et al. [2009b] measured serum lipid profiles from patients with metabolic syndrome and found that groups with different carbohydrate diets had significant differences in the serum concentration of lyso PC species and 22:6 fatty acid. Han et al. [2004a] found that modest caloric restriction of mice causes approximately 25% decrease of phospholipid mass in murine myocardium and 54 % decrease of TG in muscle. Signalling is another important application of lipidomics [Fernandis and Wenk, 2009], [Wymann and Schneider, 2008], [Serhan, 2005a]. For example, Gronert et al. [2004] found specific elevation of di-16:0 species of DAG, a product of the phospholipase D signaling pathway, in human neutrophils of patients with localized aggressive periodontitis. Also, neurolipidomics is an active field of study [Han, 2007a]. Han [2007b] found depletion of sulfatides in gray matter from subjects with Alzheimer's disease.

In case of plants, lipidomics has been applied to study the response to stress, development, gene function and food quality improvement [Wang et al., 2006], [Wolti et al., 2007].

Cellular lipidomics [van Meer, 2005] tries to answer the many open questions of cellular lipid metabolism. For example, why there are so many lipids, and how are the lipid compositions of cellular organelles determined, e.g. what is the role of metabolism vs. trafficking?

The aim of lipid fingerprinting [Han et al., 2004b], is to classify samples based on their lipid profile, for example to discriminate between bacterial species [Arnold and Reilly, 1998] or to distinguish between Fabry hemizygotes and heterozygotes from urine samples [Fuller et al., 2005]. It is not necessary to identify all the lipid species in the sample, but comparison

of the profiles or “fingerprints” is carried out using statistical methods to classify the samples.

Biomarker discovery aims to find lipids which could be used as indicators of a disease or other condition. Although it is unlikely to have a single analytical method that would allow the analysis of all lipids, due to the diversity of structures and highly different concentrations [Hu et al., 2009], LC-MS is probably the best choice for biomarker discovery. Biomarker discovery methods do not have to be quantitative, but they should be as reproducible as possible. Biomarker discovery has been applied (among others) to study LDL receptor knock-out mice [Yang et al., 2009c], benefits of fish diet [Lankinen et al., 2009a] and plant shear stress response [Han and Yuan, 2009].

In targeted lipidomics, the aim is to look for quantitative changes in specific lipids or lipid class [Taguchi et al., 2005], [Han and Gross, 2004], [Gross and Han, 2009]. For instance, lipid signalling pathways are a good targets for such studies [Serhan, 2005a].

In dynamic lipidomics [Postle and Hunt, 2009], isotope-labelled lipids or lipid precursors are used to follow the metabolism of cells [Kainu et al., 2008]. The goal is to elucidate the metabolic fluxes of the different pathways. Hellerstein et al. [1991] applied mass isotopomer analysis to the study of de novo hepatic lipogenesis. They found that the fraction of de novo synthesized palmitate was less than 2 % and stearate less than 1 % of the total palmitate and stearate in very low density lipoprotein particles, which indicates that de novo lipogenesis is a quantitatively minor pathway. The use of ESI-MS for studying the metabolism of phospholipids was pioneered by [DeLong et al., 1999] who studied the pathways of synthesis of PC using deuterated choline as the precursor. Other labels that have been used for phospholipid studies include  $D_4$ -ethanolamine,  $D_3$ -serine and  $D_6$ -inositol [Postle et al., 2004], [Kainu et al., 2008]. Isotope-labelled lipids have been used successfully to study e.g. the substrate specificity of PE N-methyltransferases [Boumann et al., 2004a] and the synthesized PC in yeast [Boumann et al., 2004b] as well as the metabolism of PC in HL60 cells [Tserng and Griffin, 2004].

## 2.6 Processing mass spectrometric lipid data

The first step of processing the mass spectrometric data is usually data conversion from proprietary format to open and well documented format like MZXML [Pedrioli et al., 2004], netCDF or even ASCII. The analysis of mass spectrometric data consist of several steps [Katajamaa and Orešič, 2005]. These typically include: 1) preprocessing, i.e. smoothing, background and/or spike removal, etc. 2) peak detection and assignment, 3) alignment, 4) peak integration, 5) de-isotoping i.e. combining the contributions of isotope peaks and reducing their overlap 6) Normalization or intensity calibration. The alignment step can correct for minor errors in the mass calibration

of the instrument and is essential for correcting the variability in the elution times of analytes in LC-MS. Depending on the experiment, various statistical methods can be used to analyze the data [Ivanova et al., 2004], [Han and Yuan, 2009], [Scholz et al., 2004].

Numerous general purpose software packages for processing MS data exist, including those provided by instrument manufacturers. There are also many software packages specifically designed for the analysis of lipid MS data, including: LipidQA [Song et al., 2007], LIPID arrays [Ivanova et al., 2004], LipID [Hubner et al., 2009], Lipid Inspector [Schwudke et al., 2006], Pecoder [Schwudke et al., 2007], Lipid Profiler [Ejsing et al., 2006], FAAT [Leavell and Leary, 2006] and MZmine 2 [Niemelä et al., 2009]. Most of the packages contain a comprehensive library of molecular formulas and isotopic distributions of lipids and some also include SMILES structures [Yetukuri et al., 2007] and/or fragmentation data [Song et al., 2007].

### 2.6.1 Isotopic distributions

Most of the elements have more than one stable isotope [Rosman and Taylor, 1998]. The isotopes have different masses due to the presence of different number of neutrons in their nucleus. The presence of isotopes is the reason that the mass spectrum of most compounds shows multiple isotope peaks [Gross, 2004]. This list of isotope peaks, each with a mass and an intensity is called the *isotopic distribution* of the compound.

Lipid molecules typically consist of  $C$ ,  $H$ ,  $O$ ,  $P$ ,  $N$ , and also  $S$  is present in sulfatides. Lipids also form adducts with  $Na^+$ ,  $Li^+$ ,  $Cl^-$  and possibly other ions.  $^{13}C$  is the most relevant isotope for lipids, since they contain many carbons and the abundance of the  $^{13}C$  is fairly large, representing 1.109% of all carbon isotopes. In case of lipidomic studies, it is generally sufficient consider the  $^{13}C$  isotope only [Han and Gross, 2001], because the error due to neglecting the isotopes of the other elements is smaller than typical experimental errors. However, if  $Cl^-$  adducts of lipids are being analyzed, the isotopic distribution of Cl must be taken into account because  $^{37}Cl$  is so abundant (24% of all chlorine).

The full isotopic distribution of a compound contains all the peaks, corresponding to the all possible combinations of isotopes that can occur in the compound. The relative intensity of each isotope peak is determined by the abundances of the isotopes and the number of each element that occurs in the molecule [Yergey, 1983]. The exact mass of a peak is the sum of the exact masses of all the isotopes from which it is composed. The *nominal mass* of a peak is the total number of protons and neutrons of its isotopes. The difference between the exact mass and nominal mass of a peak is the sum of all (positive or negative) *mass defects* of the isotopes [Gross, 2004]. The full isotopic distribution is not observed in practical measurements of biomolecules, because some of the peaks are vanishingly small and many

have such small mass difference that they cannot be resolved by the instrument.

Often the isotopic distributions of candidate (or known) compounds have to be calculated so that the peaks in a measured mass spectra can be assigned. The problem when calculating the isotopic distribution is that the number of peaks increases rapidly as the number of isotopes in the molecule increases. The common approach to overcome the combinatorial explosion is to discard and/or combine the minor peaks [Yergey, 1983], [Kubinyi, 1991], or to use Fourier transform to avoid computationally costly multiplication of the peak vectors [Rockwood and Orden, 1996]. The decision which peaks to combine in the calculation depends on the resolution of the measured spectra. When analyzing the data from unit resolution instruments, it is possible to do the calculations with nominal masses, combining the peaks with the same nominal masses, and approximate the exact masses [Rockwood and Orden, 1996]. High resolution FT-ICT instruments, with resolving power  $m/\Delta m_{50\%} > 350000$  [Hughey et al., 2001], can resolve much more isotope peaks than unit mass instruments, precluding the use of nominal mass algorithms.

One should keep in mind that the isotopic distributions depend on the isotope abundancies, which can be different e.g. in inorganic samples [Rosman and Taylor, 1998], although for lipidomics studies this rarely is the case. Notably, lipidomics studies often employ specific precursor ion scans. This will bias the observed isotopic distribution, because it represents only the part of the molecule that reaches the detector [Rockwood et al., 2003].

### 2.6.2 Data reduction

Mass spectrometric data typically consists of spectra, which are visualized as plots of intensity vs. mass/charge ratio ( $m/z$ ). The mass spectrum of a sample can be presented as a *profile spectrum*, in which there are many data points within a mass unit. Profile spectra are produced when the mass spectrometer scans the mass range, sampling the intensity of the signal produced by the ions entering the detector. Alternatively, a *centroid spectra* can be produced, where the data consists of only peak centroid masses and intensities.

One LC run can produce hundreds or thousands of individual spectra. The goal of the data processing is to reduce complexity through peak assignment, integration and deisotoping, so that a single value (total peak area) can be assigned for each compound.

The data is often preprocessed by smoothing [Andreev et al., 2003], e.g. by using median [Hastings et al., 2002] or Savitzky-Golay filtering [Yang et al., 2009a]. Baseline correction can be done by modelling approaches [Malyarenko et al., 2004], [Yang et al., 2009a] or by using an empty spectra as a point of comparison [Hermansson et al., 2005], [Wallace et al., 2004].

Before integration, the peak positions and limits have to be known. This can be done by different filtering approaches, for example wavelets [Du et al., 2006], or derivatives [Smith et al., 2006].

Peak assignment is based on mass information and can be guided by other information, like fragmentation data [Song et al., 2007], [Ejsing et al., 2006] or LC retention times [Hermansson et al., 2005]. Good mass accuracy and high resolution obviously helps in the assignment.

Deisotoping consists of 1) combining the areas of all the isotope peaks originating from the same compound, and 2) estimating the contributions of different compounds in unresolved peaks. When analyzing biological samples, the mass spectrometer often cannot resolve all the lipid peaks. In the mass spectra produced by instruments with unit resolution, the monoisotopic peak of many lipids overlaps with the second isotope peak of another lipid having one additional double bond (cf. Figure 5.8). This kind of overlap can be readily corrected for by taking into account the isotopic distributions [Kurvinen et al., 2001], [Liebisch et al., 2004], [Han et al., 2004b]. Some lipids, like cardiolipins, produce doubly charged ions and require the use of algorithms that take the higher charge state into account, such as Zscore [Zhang and Marshall, 1998]. In case of partial peak overlap, typical for the high resolution instruments, peak model fitting can be used [Meija and Caruso, 2004], [Zacarias et al., 2002], [Roussis and Prouix, 2003].

### 2.6.3 Calibration

Because of the matrix effect, differences in ionization efficiency of different lipids and selective suppression at high lipid concentrations (cf. 2.4.1), the peak intensities may not be comparable between samples. To minimize errors due to such factors, the following measures can be taken: 1) use diluted samples [Schwudke et al., 2007], 2) measure the sample at different dilutions [Zacarias et al., 2002], 3) use multiple internal standards [Brugger et al., 1997] or 4) use on-line LC to separate the lipids before MS analysis [Houjou et al., 2005].

Isotope dilution mass spectrometry, i.e. the use of an isotope-labelled analog of the analyte as internal standard, is a common method in quantitative mass spectrometry [Tai and Welch, 2004], [Briche et al., 2002]. However, it is not practical to obtain an isotope-labelled standard for each lipid present in a biological sample. Thus other means of calibration have been proposed, including the use of external standards [Pang et al., 2008], single standard for each lipid class [Ejsing et al., 2006], multiple standards for each lipid class [Hermansson et al., 2005] and multiple standards with optimal normalization factors for each detected compound [Sysi-Aho et al., 2007]. For accurate quantification, standards have to be added before lipid extraction.

Isobaric species have been quantified by utilizing fatty acyl specific fragmentations [Song et al., 2007], [Han and Gross, 2001]. Position isomers

have the same fatty acids and thus their quantification can depend only on the differences in the relative intensities of the fragment ions [Kushnir et al., 2004].



## Chapter 3

# Outline of the present study

This study consists of four publications:

1. Study of the effects of lipid concentration and structure on the instrument response in electrospray ionization mass spectrometry.
2. Development of a novel method for calculating isotopic distributions with accurate masses.
3. Development of the LIMSA software for automatic processing of mass spectra from complex lipid samples.
4. Study of the substrate specificity of secreted  $A_2$  phospholipases using electrospray ionization mass spectrometry.

## Chapter 4

# Materials and methods

PC lipid standards were obtained from Avanti Polar Lipids. Fatty acids were from Larodan AB (Malm, Sweden). Sphingosylphosphorylcholine from Matreya, Inc. (Pleasant Gap, PA).

Phospholipase D (*Streptomyces* sp.) and phospholipases  $A_2$  from cobra venom (*Naja mossambica mossambica* (P7778), bee venom (P9279), and porcine pancreas (P6534) were obtained from Sigma

All solvents were of high performance liquid chromatography (HPLC) or analytical grade and were purchased from Merck or Rathburn Chemicals Ltd. (Walkerburn, Scotland).

D3-methyl iodide was purchased from Cambridge Isotope Laboratories (Andover, MA).

Mass spectrometric measurements were done with the following instruments: 1) Esquire-LC, Bruker-Franzen Analytik, Bremen, Germany (ion trap), 2) Sciex API 300, Perkin Elmer, Massachusetts, USA (triple quadrupole) and 3) Quattro Micro, Micromass, Manchester, U.K. (triple quadrupole). Esquire-LC and Sciex API 300 were used in the first part of the work, Quattro Micro was used in the third and fourth parts.

Software development was done using ANSI C++ and Microsoft Visual Basic for Applications for Excel integration. C++ portions were compiled with Gnu Compiler Collection (gcc) and Microsoft Visual C++ 6.0 compiler.

Method	Reference
Conditions used in mass spectrometry	Papers I, III, IV
Cellular lipid extraction	[Folch et al., 1957]
Synthesis of PE or PS species from PC by PLD transesterification	[Kasurinen and Somerharju, 1995]
Synthesis of $D_9$ -labelled PC from PE by $D_3$ -methyl Iodide	Paper IV
Synthesis of SM standards	[Koivusalo et al., 2004]
Synthesis of PC series with systematically varying $sn_1/sn_2$ chain	[Somerharju et al., 1987]
Lipid purification using HLPL on a diol column	[Silversand and Haux, 1997]
TLC separation of phospholipid classes	Paper I
Determining phospholipid concentration by phosphorous assay	[Bartlett and Lewis, 1970]
Assay of $PLA_2$ specificity with octyl glucoside micelles	Paper IV
Assay of $PLA_2$ specificity with unilamellar vesicles	Paper IV

Table 4.1: Methods used during the course of the work

# Chapter 5

## Results and discussion

### 5.1 Behaviour of phospholipids in the mass spectrometer

At the time this work was initiated, no systematic studies addressing quantitative analysis of phospholipid compositions by ESI-MS had been published. Accordingly, we set out to study the effect of lipid concentration and structure on the instrument response [Paper I].

#### 5.1.1 Effect of PC acyl chain length and unsaturation

We analyzed mixtures of phosphatidylcholine (PC) standards at different concentrations. Figure 5.1 shows how the intensity of the measured peaks varies with phospholipid concentration (panel A). The intensities of the different PC species appear to increase linearly at low concentration, but level off at higher concentrations. This kind of signal saturation is typical for ESI-MS and presumably due to limiting charge density at the surface of the spray droplets [Enke, 1997]. Notably, however, the *relative* intensities of the different PC species remain linear up to a higher,  $5 \mu M$ , concentration range (panel B). This makes it possible to determine the concentration of analytes even at high concentrations by comparing their intensities to those of internal standards.

Figure 5.2 shows how the instrument response depends on the combined length (carbon number) of the acyl chains of PC as shown previously by Brugger et al. [1997]. The decrease in intensity with chain length is significantly more than what is to be attributed to the decrease of the relative size of the monoisotopic peak and is probably due to decrease in the surface activity of the PC molecules with increasing chain length. At high total concentration, the surface charge density becomes limiting and the more surface-active short-chain PC species can outcompete the less surface active long chain ones. Beside this effect, differences in the vaporization en-

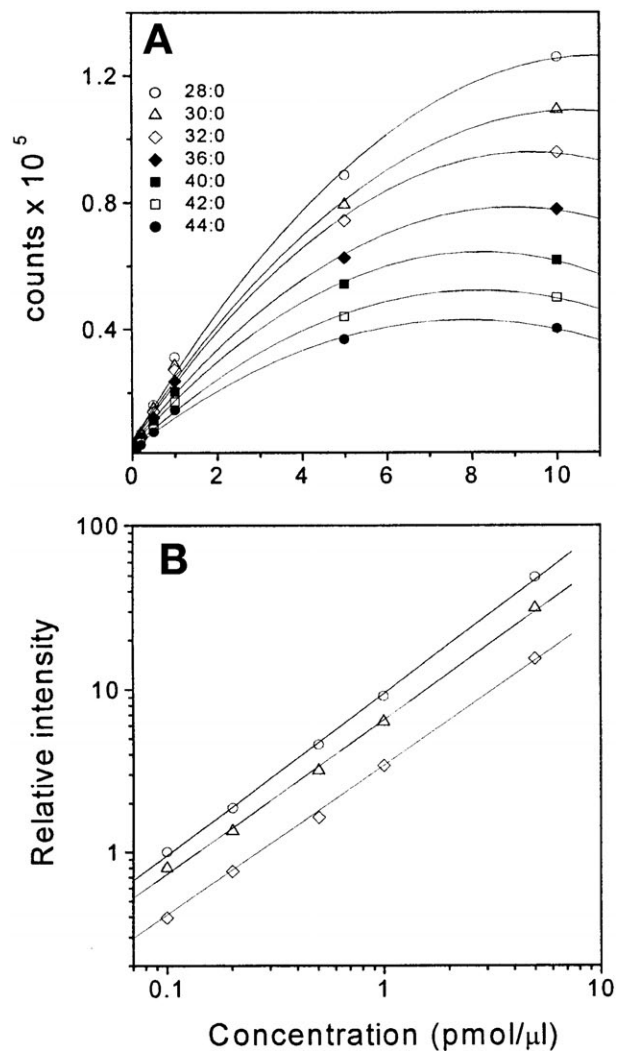


Figure 5.1: Effect of phospholipid acyl chain length and concentration on instrument response. The samples contained seven saturated PC species with total acyl chain length from 28 to 44 at different dilutions (0.1 - 10  $\mu M$ , or 0.7 - 70  $\mu M$  total lipid concentration) and PC 26:0 whose concentration remained constant 0.1  $\mu M$ . Panel A: Peak intensity vs. concentration. Panel B: Response for selected species relative to that of the 26:0 species. ○ = 28:0, △ = 36:0, ◇ = 44:0.

ergy may also contribute to the chain-length dependency of response [Enke, 1997]. The chain length effect is linear at under 5 pmol/ul, but at high concentration regime the (see figure 5.2) it becomes nonlinear.

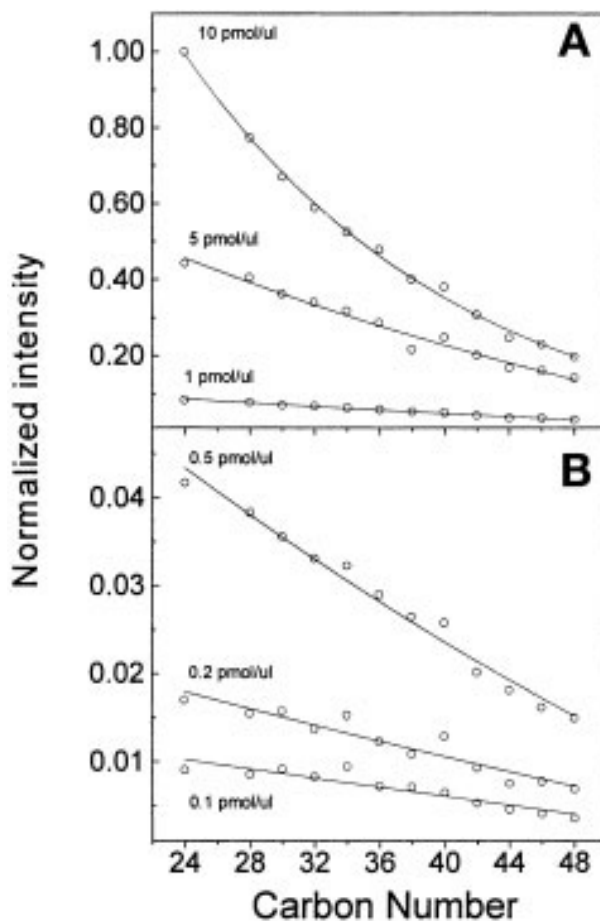


Figure 5.2: Instrument response vs. PC carbon number at different concentrations. An equimolar mixture of 12 saturated PC species with different acyl chain lengths at different dilutions (0.1 - 10  $\mu M$  per species, or 1.2 - 120  $\mu M$  total lipid concentration). Panel A: Normalized data for the three highest concentrations. Panel B: data for the three lowest concentrations. Data normalized to the highest intensity value.

To study the effect of acyl chain unsaturation on the instrument response, different concentrations of an equimolar PC mixture containing five 36-carbon species with 0, 1, 2, 4 or 6 acyl chain double bonds was analyzed (Figure 5.3). At high concentrations, the instrument response increased significantly with increasing number of double bonds, but at lower concentrations this effect diminished and eventually disappeared. Similar results have been obtained

previously for triacylglycerides [Duffin et al., 1991]. Double bonds reduce the hydrophobicity of the acyl chains, which could increase the affinity of the lipid to the surface of the solvent (chloroform-methanol) droplets.

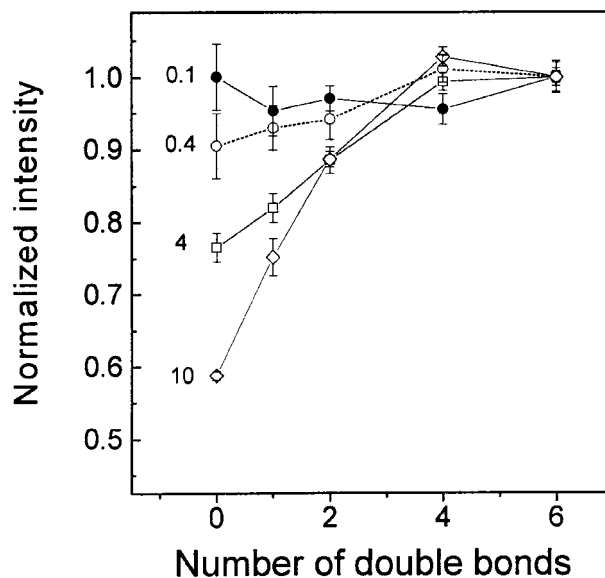


Figure 5.3: Effect of the number of double bonds. Lipid concentration per species: ● = 0.1  $\mu M$ , ○ = 0.4  $\mu M$ , □ = 4  $\mu M$ , ◇ = 10  $\mu M$ . The response values have been normalized relative to the average of the response for the 36:6 species at each concentration. The error bars indicate the standard deviation ( $n = 5$ ).

### 5.1.2 Effect of polar head group and ammonia

As expected, the polar head group and ionization mode have a major effect on the ionization efficiency of phospholipids (Figure 5.4). In the negative ion mode, the highest response was observed for PG, followed by PI, PA and PS. PE and the chloride adduct of PC gave a much smaller response. Inclusion of ammonia (1 %) in the infusion solvent markedly increased the response for PE and PA relative to other negatively charged phospholipids (Panel C).

In the positive ion mode, in the absence of ammonia, the sodium adducts of PC and PE were prominent (Panel D), which complicates the analysis. However, when ammonia was included, the relative intensities changed dramatically (Panel F) so that the intensity of protonated PC far exceeded those of the sodium adducts.

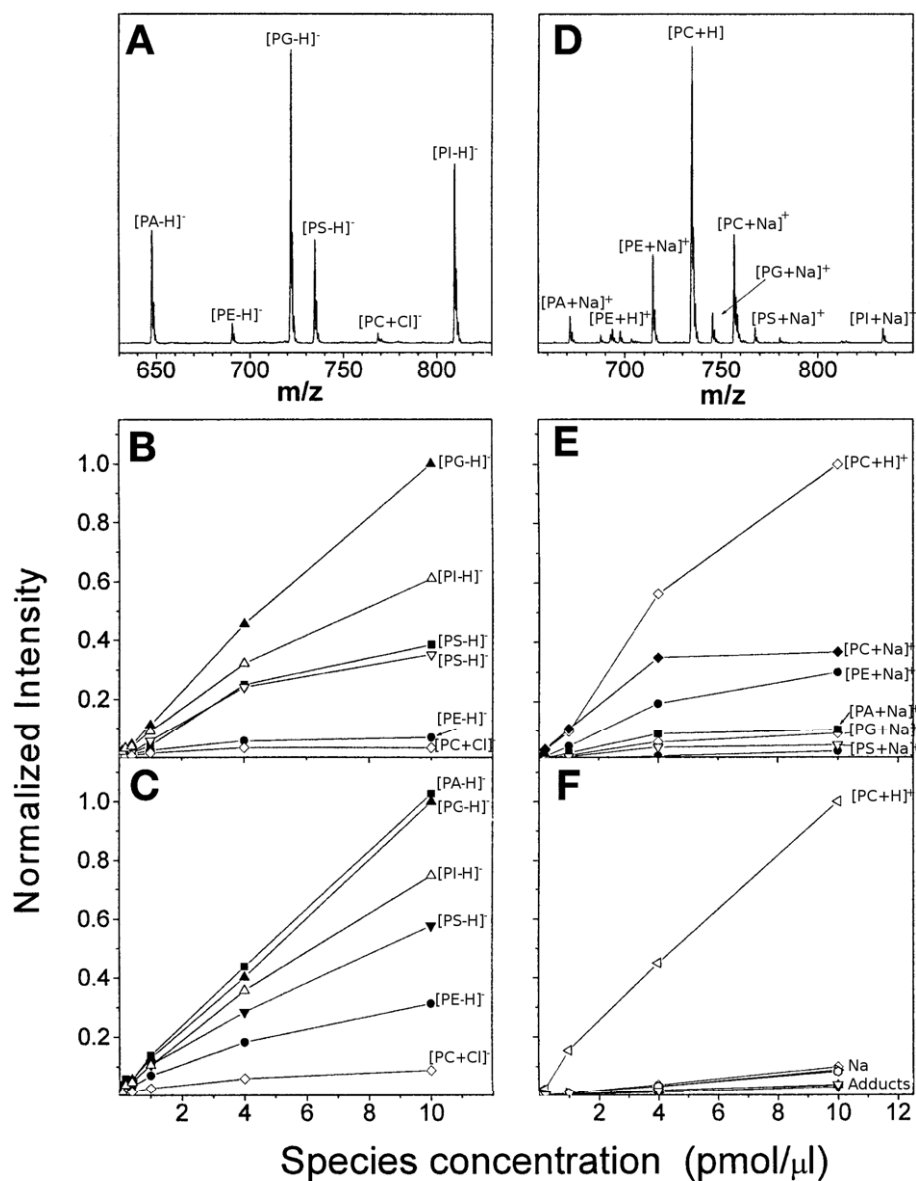


Figure 5.4: Effect of head group at different concentrations. An equimolar mixture of dipalmitoyl-PC, -PE, -PS, -PG, -PI and -PA was analyzed at various dilutions. Panel A: Negative-ion spectrum at 10  $\mu M$  per species. Panel B: Relative instrument response vs. concentration in negative mode without added ammonia. Panel C: As in B, with added 1%  $NH_4OH$ . Panel D: Positive-ion spectrum at 10  $\mu M$  per species. Panel E: Relative instrument response vs. concentration without added ammonia. Panel F: As in E but with added 1%  $NH_4OH$ .



### 5.1.3 Implications on phospholipid quantification

Beside lipid structure and solvent composition, the instrument response is also sensitive to instrument settings. The parameters should be set to e.g. optimize analyte ionization and minimize cone fragmentation. In MS/MS analyses, the collision energy is important and can be programmed to increase with analyte molecular weight to obtain similar response [Ekroos et al., 2003]. However, care should be taken not to compromise the detection of species in the low or high molecular mass range.

As shown in Figs 5.2 and 5.3, dilution of the sample can reduce the lipid structure -dependent differences in instrument response. However, there is a limit to sample dilution below which the lipids present at low concentrations (like minor PS, PA and PG species) cannot be reliably detected or quantified, particularly, if detergents or other surface-active suppressors of ionization are present.

Internal standards need to be included for each lipid class to account for the structure-dependent differences in ionization efficiency. While the accuracy of quantification increases with the number of internal standards, the availability or cost of the latter often limits their number to 1-3. When using more than 1 standard per class the (often unpredictable and biasing) suppression effects caused by salts or other impurities can be observed and partially eliminated. The standards should be added before extraction to account for possibly different recoveries of different lipids. We have found that diunsaturated standards are most useful as their response is similar to that of the (typically unsaturated) biological lipids.

## 5.2 Method for calculation of isotopic distributions with accurate masses

Knowing the isotopic distributions of compounds is necessary for being able to assign compounds to peaks found in mass spectrometric data. Isotopic distributions are also needed in isotopomer analysis, where one aims to deduce the position of the stable isotope from the fragment data, possibly allowing to distinguish between alternative biosynthetic pathways [Hellerstein and Neese, 1999].

Various methods for computing isotopic distributions have been proposed [Yergey, 1983], [Kubinyi, 1991], [Rockwood and Orden, 1996]. These methods either trade mass accuracy for speed of execution or approximate the masses. We developed method for calculation of isotopic distributions with good mass accuracy for unit resolution mass spectrometers [Paper II].

Our method has the advantage to previous methods that it is relatively easy to understand and computationally efficient, while still maintains very high accuracy.

Our method does not calculate the fine isotopic structure that the high resolution instruments can partially resolve. Algorithms that can calculate high resolution isotopic distributions [Rockwood et al., 1996] [Snider, 2007] are necessarily slower and unnecessary for processing data from unit mass resolution instruments.

The isotopic distribution calculation algorithm in Paper II breaks the calculation into a binary series of convolutions, similar to the method reported by Rantanen et al. [2002], with the number of atoms involved in the calculation doubling at each successive iteration. In each convolution step (Figure 5.5), a Cartesian product of the isotopic distributions is formed so that the corresponding peak intensities are multiplied and masses added together (Step 1). To keep the algorithm efficient, all the peaks having the same nominal mass are combined by using weighted average (Step 2). This merging reduces the amount of calculations dramatically while still keeps good mass accuracy.

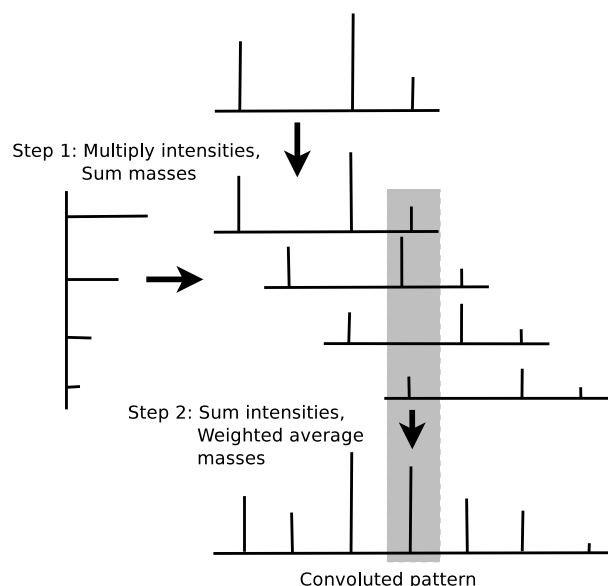


Figure 5.5: Principle of the peak convolution algorithm. Two isotopic distributions are convoluted to form a larger isotopic distribution. In step 1, all the peak intensities are multiplied and masses added together to obtain the peaks in the convoluted pattern. In the step 2, all the peaks having the same nominal mass are combined by adding together their intensities and calculating the mass of the new peak by weighted average.

Molecular formula	Average mass (Da)	Number of peaks	Average absolute mass difference (Da)
$(CH_2O)_{100}$	3002.59	18	1.19587e-06
$(C_{39}H_{49}N_{15}O_{24}P_4)_{100}$	123578.57	105	0.000142669
$Sn_{1000}$	118710.00	884	2.79372e-07
$C_{44}H_{87}NPO_8$	789.13	11	2.54449e-07

Table 5.1: Comparison of the mass accuracy of the program from Paper II and qmass. Qmass uses the Fourier transform method for calculating the isotopic distributions [Rockwood and Orden, 1996] and another algorithm for calculating the isotope compositions and accurate masses of isotopic peaks [Rockwood et al., 2004]. Pruning limit used in the calculation with the program from Paper II was  $1e-30$ . Only the nominal isotopic peaks that were larger than 1 ppb of the largest peak in the isotopic distribution were used for calculating the average absolute mass difference between the two programs.

### 5.2.1 pruning

If the unit resolution isotopic distributions are not pruned, they grow proportionally to the number of atoms in the molecule, and because convoluting the distributions is computationally expensive, the algorithm would be slow for large molecules. However, the peaks at the sides of the isotope distributions of large molecules are vanishingly small. By pruning (removing) these peaks, the size of the distributions does not grow unduly. Because the pruning only occurs at the edges of the isotopic distribution, and when the pruning threshold is set low, the mass accuracy of the calculated isotopic peaks is high and agrees with other program that uses unrelated algorithms (Table 5.1 to sub ppb level).

## 5.3 Automated analysis of complex lipid samples

LIMSA is a program (Excel add-on) that was developed to simplify quantitative analysis of complex lipid mixtures [Paper III]. LIMSA was designed for the analysis of MS spectra obtained in direct infusion experiments carried out with unit resolution instruments. Many other software packages exist for lipid analysis (See Chapter 2.6). The choice of the package depends on the type of the instrument producing the spectra, the type of experiments (e.g. direct infusion vs. LC-MS) and types of lipids in the analysis. Some packages are specifically developed for the analysis of spectra from high resolution instruments (e.g. FAAT [Leavell and Leary, 2006], LipID [Hubner et al., 2009] and Pecoder [Schwudke et al., 2007]), whereas others are developed for unit resolution instruments (LIMSA [Paper III], MSPEC-

TRA [Kurvinen et al., 2001] and LIPID arrays [Ivanova et al., 2004]), or can handle both kinds of data (MZmine 2 [Niemelä et al., 2009]). Lipid Profiler [Ejsing et al., 2006], LipidQA [Song et al., 2007] and Lipid Inspector [Schwudke et al., 2006] are developed for data from hybrid quadrupole - time of flight instruments, although some of them can also process spectra from triple quadrupole instruments. There are packages designed for LC-MS data (LipID, MZmine 2 and [Hermansson et al., 2005]). Some also include statistical analysis tools: LIPID arrays for comparative experiments and MZmine 2 for e.g. principal component analysis. MZmine 2 can also be extended with custom analysis modules.

LIMSA has an internal database of thousands of lipids and it carries out the necessary steps required for identification and quantification of the lipids in complex mixtures. LIMSA's lipid database is user extensible and compatible with isotope-labelled lipids. The user can specify the molecular formula of the fragment, which may contain an isotope label, in specific scan modes (precursor ion or neutral loss) and LIMSA will calculate the correct isotopic distributions for these modes. This ability makes LIMSA especially suitable for analysis of data from isotope-labelled metabolic experiments. Figure 5.6 shows the steps of data processing by LIMSA. LIMSA works per spectrum basis, necessitating the use of other tools for either combining or extracting parts from multi-spectra datasets, and for statistical analysis of the results from individual LIMSA runs.

For example, Figure 5.7 shows spectra obtained from an isotope labelling experiment. LIMSA can be used in batch mode allowing automated analysis of multiple spectra which is useful when constructing e.g. time series graphs (lower panel).

### 5.3.1 Deisotoping

LIMSA employs three alternative algorithms for deisotoping of mass spectra: 1) a subtraction algorithm, 2) a linear fit algorithm and 3) a peak model fitting algorithm. The subtraction algorithm subtracts the scaled theoretical isotopic distribution of the compound with the lowest  $m/z$  from the spectrum, thus eliminating the contribution of this compound. The procedure is repeated for the next compound until no further compounds remain. This type of algorithm is the most commonly used one in lipid analysis software (Han and Gross [2004], Kurvinen et al. [2002], Liebisch et al. [2004]).

The linear fit algorithm models the overlapping peak intensities as a weighted sum of isotopic distributions using a set of linear equations, which is solved to yield the contributions of the individual molecules [Meija and Caruso, 2004]. In LIMSA, the algorithm is constrained to positive weights in order to increase performance with noisy data.

The peak model fit algorithm [Meija and Caruso, 2004] used in LIMSA models the raw spectrum as a weighted sum of isotopic distributions with

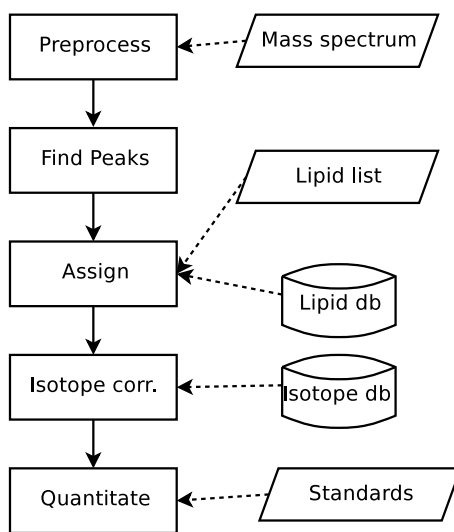


Figure 5.6: Lipid MS data processing steps carried out by LIMSA. The user supplies the mass spectra, the list of lipids that may be present in the sample and the list of standards. LIMSA picks the peaks present in the spectrum and uses its database of lipid molecular formulas and isotope distributions to calculate the isotopic distributions of lipids and uses them for assigning and de-isotoping the peaks. LIMSA uses the standards for data normalization/quantification.

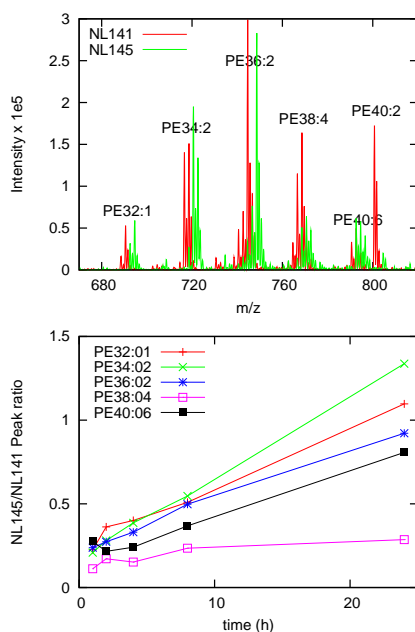


Figure 5.7: Analysis of the data from a heavy-isotope labelling experiment using LIMSAs. The PE species of HeLa cells were labelled by including D4-ethanolamine (100 g/ml) in the cell culture medium for up to 24 h. Upper panel: Mass spectra obtained for the cellular lipid extract using neutral loss of 141 (selective for the unlabelled PE species) and neutral loss of 145 (selective for the D4-labelled PE species). PE 40:2 is an unlabelled internal standard and thus no corresponding labelled species is seen. Lower panel: Time series constructed from the output of multiple LIMSAs runs showing the ratio of labelled to unlabelled PE molecular species with time.

a gaussian peak shape (Figure 5.8). If the peak model parameters are constant, linear equations could be used. However, LIMSA allows the peak width to vary and the isotopic distributions to move along the  $m/z$  axis, thus combining peak modeling and alignment. This nonlinear fitting problem is solved using the Levenberg-Marquardt algorithm implemented in the Gnu Scientific Library ([www.gnu.org/software/gsl/](http://www.gnu.org/software/gsl/)). Since nonlinear fitting tends to be very sensitive for the initial choice of fitting parameters, LIMSA first carries out preliminary gaussian filtering -based peak finding, alignment and integration steps to obtaining good starting values for the optimization routine.

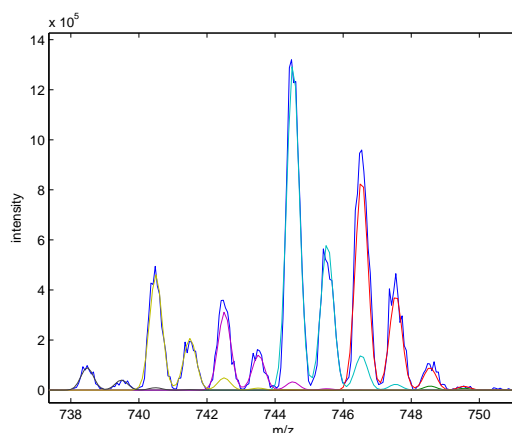


Figure 5.8: Overlapping PE species. Dark blue trace: Neutral loss 141 spectrum. Black, yellow, magenta, light blue, red and green traces: the calculated neutral loss 141 isotope distributions for PE36:5, PE36:4, PE36:3, PE36:2, PE36:1, PE36:0, respectively. Fitted to the data using the peak model deisotoping algorithm of LIMSA.

Comparison of three algorithms for isotopic distribution deconvolution (deisotoping) showed that the subtraction and linear fit algorithms performed comparably in terms of accuracy while the peak model fit algorithm performed best both with mixtures of standards and human HDL lipid extract. Especially with less well resolved spectra, peak model fitting was superior as shown in Figure 5.9. The results obtained with linear fit algorithm were unacceptable at low spectral resolution. As tested with lipid standards, the limit for detection (at CV 25 %) of a minor signal was approximately 10 % of the intensity of the major overlapping signal.

When the signal to noise ratio was high and the spectral resolution adequate, all three deisotoping methods produced similar results. The peak model method was 10-100 times slower the other two methods, but generally this is irrelevant regarding the throughput of lipid analysis by MS.

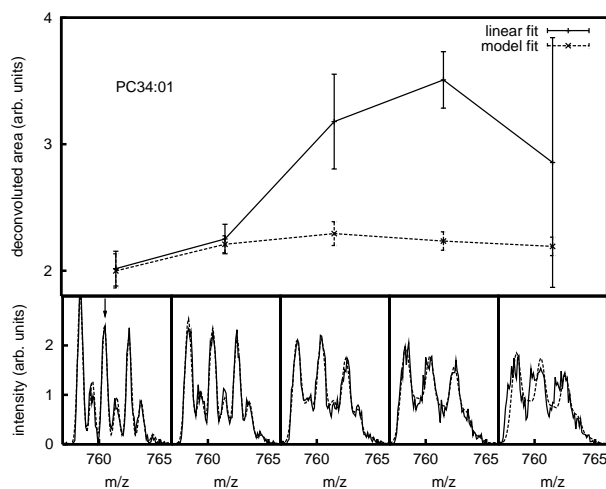


Figure 5.9: Comparison of performance of the linear fit and peak model fit deisotoping algorithms with three compounds (PC34:2, PC34:1 and PC34:0) and five different instrument resolution settings. Lower panels: representative spectra. Upper panel: deconvoluted peak area of the middle compound (PC34:1). Error bars represent the standard deviation ( $n = 8$ )

Unlike the subtraction algorithm, the linear and the peak model fit algorithms can deconvolute isotopic distributions even when all the peaks overlap, provided that the distributions themselves are different enough [Meija and Caruso, 2004]. Such a situation can arise for example when a partially isotope-labeled (impure) standard overlaps a sample lipid.

### 5.3.2 Normalization

LIMSA can quantify the lipids in biological samples if one or more internal standards per lipid class is included. In case of several standards, LIMSA fits a simple linear regression model to the standards to get an estimate of the instrument response at each point on the  $m/z$  axis. LIMSA assumes that the instrument response depends on the molecular weight for each lipid class. LIMSA does not model the effect of double bond to the instrument response, although separate standards can be used for saturated and unsaturated lipids. Nonlinear function for modeling the molecular weight - dependent instrument response could also be used, but in our experience, this does not improve the accuracy significantly.

To test the quantification by LIMSA, test mixtures with different PCs with known concentrations were made. PC-32:2, PC-40:2 and PC44:2 were chosen as internal standards and their concentration was kept constant  $2 \mu M$ . The concentration of the other PC:s ranged from 0.01 to  $5 \mu M$ . Figure



5.10 shows the known vs. measured concentration for select analyte PCs. As can be seen, some of the PC:s (28:0 and 40:8) are overestimated, some (38:0) are underestimated and some (36:4, 36:1 and 40:6) are measured accurately. The use of diunsaturated standards slightly underestimates the concentration of saturated PC species. The mean error in the measured concentrations was 15 %. When using a few internal standards it is not possible to fully correct for the differences in response due to varying acyl chain unsaturation. However, the errors are generally small if one employs diunsaturated standards as they give similar responses as most biologically relevant phospholipid species.

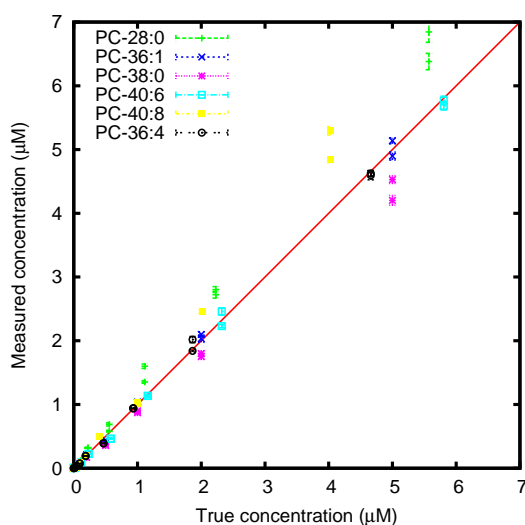


Figure 5.10: Test of accuracy of quantification with LIMSAs. The red line shows the ideal quantification. The data are from two separately prepared sets of the PC test mixtures. Symbols denote the mean and the 95 % confidence intervals for the mean of 8 measurements

Beside accuracy, the sensitivity of analysis is of interest. Figure 5.11 shows the coefficient of variation vs. the concentration of PC. If the CV threshold is set to 0.2, approximately  $0.01 \mu M$  PC can be reliably quantified.

## 5.4 Substrate specificity of phospholipases

Traditionally, phospholipase specificity has been studied using radiolabelled phospholipid substrates [Sundler et al., 1994]. This approach is not practical when comparing many different phospholipid substrates as each of them have to be studied separately. This introduces problems as the curvature of the substrate vesicles or the enzyme affinity to the substrate may differ

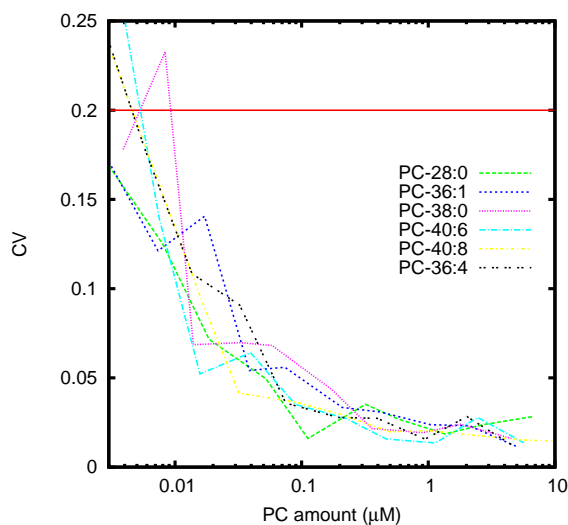


Figure 5.11: Sensitivity of phosphatidylcholine quantification by LIMSA. The red line marks the 0.2 CV threshold for reliable quantification.

between the experiments. Two lipids can be studied simultaneously if they are labeled with different radioisotopes ( $^3H$  and  $^{14}C$ ) [Ghomashchi et al., 1991]. This improves the accuracy of the assay as the other lipid can be used in all substrate mixtures, but this approach as well is impractical when studying the hydrolysis of many phospholipids.

Due to its high resolving power, mass spectrometry can be used to study simultaneously the hydrolysis of a multitude of phospholipids. This approach was first implemented by Kuksis and coworkers who studied the hydrolysis of the phospholipids in native lipoproteins by secretory phospholipase [Pruzanski et al., 2005], [Pruzanski et al., 2007]. We used a similar approach to study the hydrolysis of tens of different phospholipid species by A-type phospholipases (PLAs) in micelles or small unilamellar vesicles (SUVs) [Paper IV]. After addition of PLA, timed samples were taken from the reaction mixture and concentrations of the individual phospholipid species were determined by mass spectrometry. Figure 5.12 shows the normalized concentrations of select PC species with time and major differences in the rate of hydrolysis are obvious.

#### 5.4.1 Analysis of kinetics

The mass spectrometric data was first processed by LIMSA, to obtain the normalized peak areas. The kinetics of the hydrolysis were then obtained by least squares fitting a first order progress curve. The maximum fraction of the hydrolyzable substrate was set to that accessible to the enzyme, i.e.

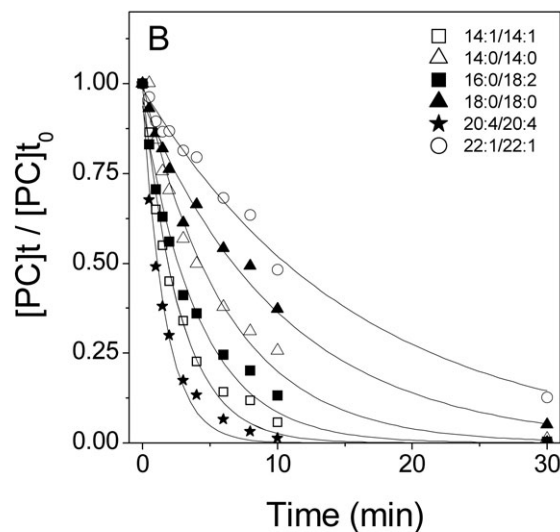


Figure 5.12: Hydrolysis of select PC species in micelles containing 25 different PC species and sphingomyelin 18:0. First order reaction progress curves fitted using gnuplot software (<http://www.gnuplot.info/>)

0.67 for SUVs (only the molecules in the outer layer are accessible) and 1.0 for micelles (all of substrate is accessible).

During simultaneous reaction the conditions of the experiment (i.e. the enzyme concentration) stay the same for all substrates and assuming that all PC molecules are equally accessible (which is certainly valid for micelles but not necessarily for vesicles due to possible differences in transbilayer distributions, the relative reaction velocities (substrate specificities) are obtained directly from the ratio of the normalized reaction velocities [Fersht, 1985], [Duggleby, 1995]. For first order reaction, the normalized reaction velocity equals  $-k$  (the rate constant).

#### 5.4.2 Specificity of three secretory PLAs

We studied the specificity of three different PLA enzymes, i.e. those from cobra and bee venom and porcine pancreas  $PLA_2$ . The substrate consisted of a mixture of PCs and also included 5 mol% of phosphatidic acid to provide negative charge increasing the affinity of the enzymes for the macrosubstrate (micelle or SUV).

Figure 5.13 shows the relative rates of hydrolysis by the three PLAs of PC species with varying acyl chain length and unsaturation. In micelles (left column) the rate of hydrolysis decreased modestly with increasing chain length for both the bee and cobra enzymes. The rate of hydrolysis by the

bee enzyme increased markedly with increasing acyl chain unsaturation, and the arachidonic acid containing PC:s were hydrolyzed especially quickly. In contrast, with the cobra  $PLA_2$ , unsaturation had hardly any effect, except for the PC with a 22:6 or 18:3 chain. The porcine  $PLA_2$  favored short di-unsaturated species as well as the 16:0/18:2 and di-18:3 species. These data suggest that the acyl-binding sites of the different PLAs differ significantly.

The specificity profiles obtained with SUVs differed markedly from those for micelles (Figure 5.13 right column). Most notably, the length of a saturated acyl chain had a far stronger effect on the rate of hydrolysis in SUVs. However, the effects of double bond position and number were similar to those found for micelles.

To obtain more detailed information on the contribution of the individual acyl chains, we synthesized two sets of saturated PC species in which the length of either the *sn1* (the C<sub>n</sub>/16:0-PC series) or *sn2* (the 16:0/C<sub>n</sub>-PC series) acyl chain varied from 6 to 24 carbons, while the length of the chain in the other sn-position was kept constant at 16 carbons. The C<sub>n</sub>/16:0-PC species contained a  $D_9$ -labeled choline headgroup, whereas the 16:0/C<sub>n</sub>-PC species were unlabeled. Both PC sets were present in the hydrolysis reaction simultaneously, which allows one to determine the relative rates of hydrolysis of positional isomers accurately. The  $D_9$ -label is unlikely to affect the hydrolysis because the PLAs studied are not specific to the polar head group of phospholipids and the deuteriums are too far from the hydrolyzed bond to cause significant kinetic isotope effect.

Figure 5.14 displays the hydrolysis of both the C<sub>n</sub>/16:0- and 16:0/C<sub>n</sub>-PCs. With all the PLAs the rate of hydrolysis decreased monotonically as the length of the sn1 acyl chain increased. In contrast, the effect of the -sn2 chain length was complex. With the bee enzyme, increasing of the length of this from 6 to 8 carbons increased the rate of hydrolysis, which then decreased sharply at carbons 9-11 and finally leveled off. The cobra enzyme behaved similarly but showed an additional (modest) peak at 13-14 carbons. The porcine  $PLA_2$  discriminated between positional isomers even more effectively than the other enzymes. For example, 16:0/9:0-PC was hydrolyzed 9-fold faster than its 9:0/16:0 isomer. These findings suggest that the sn2 chain associates more intimately with the enzyme than the sn1 chain, which is consistent with the crystallographic data obtained for cobra  $PLA_2$  complexed with a non-hydrolyzable PC analogue [Scott et al., 1990]. Peaking of the activity of the bee enzyme when the length of the sn2 chain is 9 carbons indicates that this equals the dimension of the sn2 chain binding cavity of this enzyme, in agreement with previous crystallographic data [Thunnissen et al., 1990].

In vesicles, the rate of hydrolysis by bee and cobra PLAs (the pancreatic enzyme was inactive with vesicles) diminished much more with increasing chain length than in micelles (Figure 5.15) and remarkably, there was practically no difference between sn1 and sn2 isomers, in contrast to what was

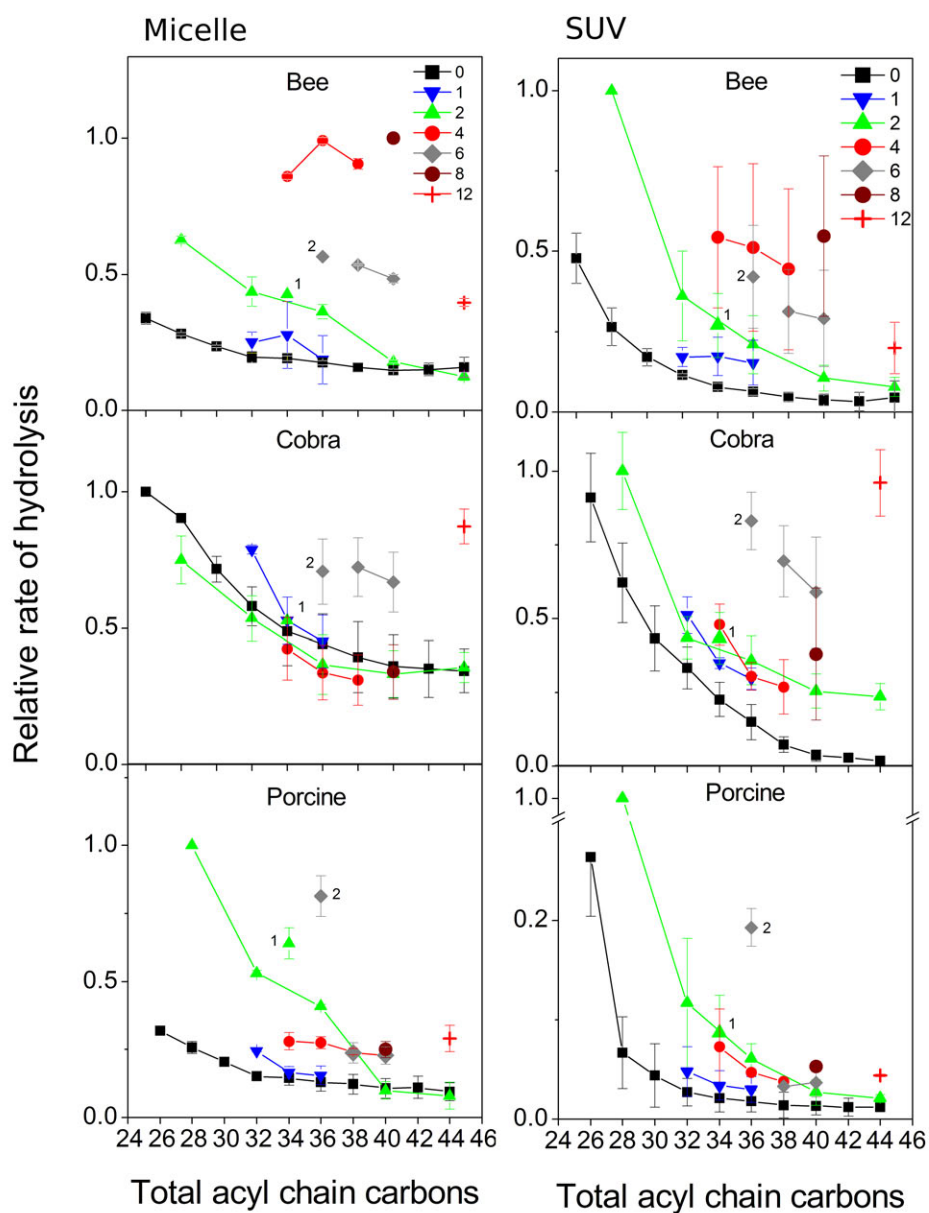


Figure 5.13: Relative rates of hydrolysis of PC species by bee, cobra and porcine  $PLA_2$  in micelles and SUVs. Rates are normalized to the fastest species. PCs are grouped to series according to the total number of double bonds in the acyl chains (see Legend). 1 and 2 indicate 16:0/18:2 and di-18:3 species that are not homologous with the other species marked with the same symbol.

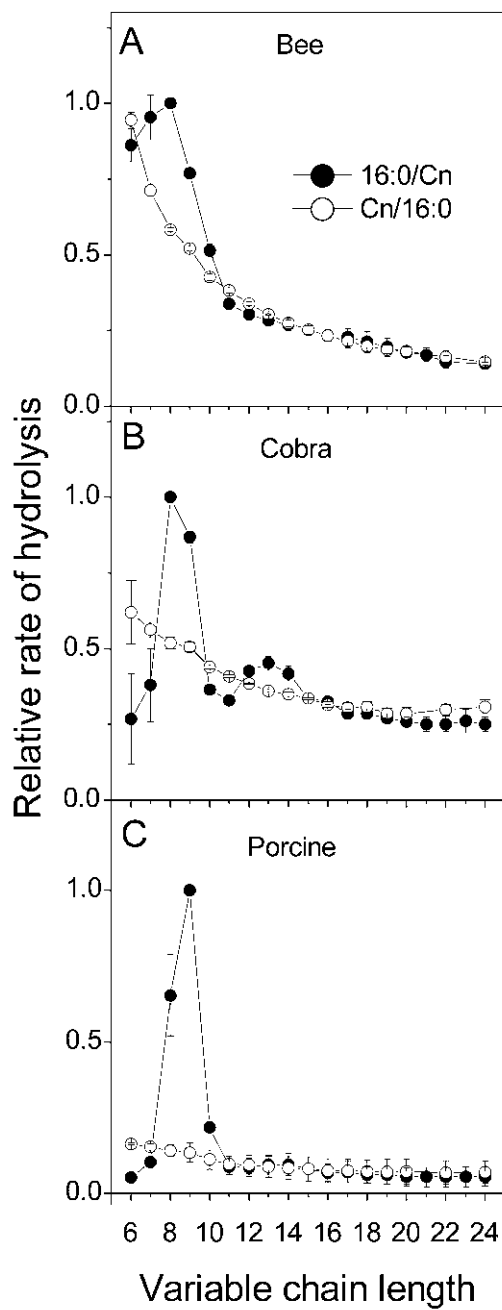


Figure 5.14: Hydrolysis of acyl chain positional isomers of PC in micelles. See text for details.

observed for micelles.

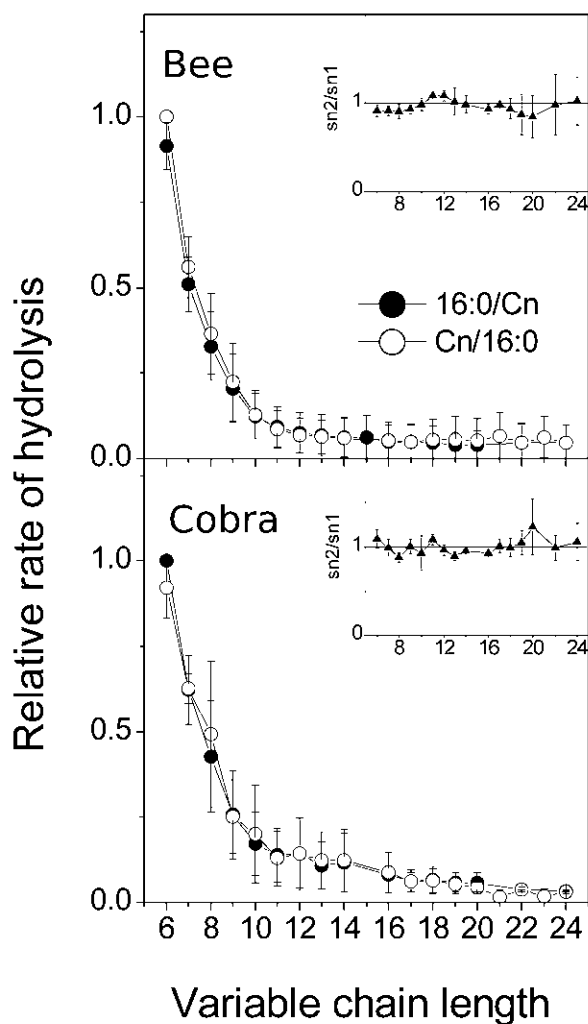


Figure 5.15: Hydrolysis of acyl chain positional PC isomers by bee and cobra PLAs in SUVs. See text for details.

The very strong effect of the acyl chain length (molecular hydrophobicity) and lack of discrimination between positional isomers in vesicles strongly suggests that the efflux of the phospholipid substrate is a key factor in PLA specificity. Previous studies with cross-linkable phospholipids support this notion (Soltys et al. [1993], Wu and Cho [1993]). However, accommodation of the substrate acyl chains in the binding site of the enzymes also plays a role in specificity of the secretory PLAs as indicated by differences in the hydrolysis of unsaturated PCs or positional isomers in micelles by the three

enzymes (Figures 5.13 and 5.14).

In summary, our findings provide strong evidence that substrate efflux propensity is a key player in selective hydrolysis of membrane-bound substrates by secretory PLAs.



# Acknowledgements

I wish to thank my supervisor Pentti Somerharju for support and guidance, Andreas Uphoff for encouragement and help, Martin Hermansson for critical comments and hard work with experiments and manuscripts, Krishna Batchu for a positive attitude and persistence with the experiments and Tarja Grundstrm for expert assistance.

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