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**Comparison of chromatographic separation
methods in lipidomics: with or without HPTLC**

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This work has developed during my external practice in COS (Centre for Omic Sciences), which is situated in avinguda universitaria nº1, Reus and their webside is <http://omicscentre.com/>.
It has been supervised by Núria Canela and Sílvia Mariné.

Table of contents

ABSTRACT.....4

HYPOTHESIS AND OBJECTIVES.....4

INTRODUCTION.....5

MATERIALS AND METHODS.....16

RESULTS.....19

DISCUSSION.....27

CONCLUSIONS.....28

ACKNOWLEDGEMENTS.....28

BIBLIOGRAFY.....29

Abstract

Lipodomics is a booming science. It's a part of metabolomics and it consists in lipid analysis. It develops in three steps: extraction, separation and analysis of lipids. For chromatographic separation we used HPTLC and HPLC and for analysis we used Q-TOF and QqQ. Phospholipids are a type of lipid that have important functions such as the formation of cell membranes, signal transmission or the formation of lipoproteins. Our objective was to determine if the application of HPTLC before HPLC separation implies an improvement in the detection of phospholipids. We used various working conditions: five different extractions, eight mobile phase for HPTLC, with and without derivatization process and using a volume of 5µl and 50µl in a HPTLC plate. We have concluded that it is not necessary because the results for analysis are the same.

Hypothesis and objectives

The hypothesis is: phospholipid separation would be more efficient with HPTLC separation after analysis than without HPTLC.

The main objective of this work was compare results in a separation of phospholipids obtained from human serum using HPTLC technique prior to HPLC or without applying it. The other objective was testing HPTLC with different mobile phases, volumes and extractions.

Introduction

1.Omic sciences¹

Omic sciences are described as the study of DNA, RNA, proteins and metabolites in its entirety. The most common omic sciences are Genomics, Transcriptomics, Proteomics and Metabolomics. The suffix "-omic" comes from Latin and means "whole."

Genomics aims to study the genome, which is a total DNA from a cell. Transcriptomics studies the transcriptome, which is the DNA that is transcribed to RNA. Proteomics is responsible for analyzing the all proteins of an organism, called proteome, while metabolomics studies the metaboloma present in a biological system at a particular time. Omic sciences are part of Systems Biology, which studying the biological system together, instead of observing various parts in isolation.

1.1 Metabolomics

Metabolomics is the study of metabolites present in the organism. A metabolite is a small molecule which is a result of the food metabolism. The metabolome is the set of these metabolites. Metabolites are present in processes such as cell signalling, energy transfer and the diseases development.

Metabolomics is used for dynamic studies and to make predictions about the state of the body. There are several ways in metabolomics assays: by NMR, by gas chromatography (GC) or liquid chromatography (LC) coupled to mass spectrometer (MS).

The applications of Metabolomics are biomarkers discovery, clinical trial testing, toxicity assessment/toxicology testing, food and beverage yeasts, host-microbial interactions or environment.

1.1.2 Lipidomics

Lipidomics is a part of Metabolomics, and studies the lipid profile of an organism, which is called lipidoma. The studies of lipidomics begin with the extraction of lipids from biological material. Afterwards, you can analyze the sample in MS or you can make a prior chromatographic separation.

Depending on the objectives of the study, there are two types of Lipidomics: Target lipidomics and Non-Targeted Lipidomics. The Target Lipidomics consist in detecting and/or quantifying metabolite previously identified, whereas Non-Targeted refers to what type of lipids we found in a sample.

2. Lipid extraction¹

The extraction of lipids is an essential part in Lipidomics because it determines which lipids will be analysed in the future. There is no standard protocol: each extraction is determined by the type of sample, analyzed lipid, etc. Most extractions are based on the formation of two phases, the aqueous phase and the organic phase. The organic phase containing our lipids, and the aqueous phase contain the rest of metabolites.

It is essential to extract the lipids from the sample because the mass spectrometry is a very sensitive device. The sample from the tissue must be homogenized previously. We prove different extractions such as Bligh & Dyer method, Extraction with MTBE, liquid-liquid extraction using butanol, Extraction with OSTRO plate and a method found in the literature exclusive for phosphatidyl cholines in our experiment. The procedures are described in "Materials and methods".

3. Lipid separation: chromatography¹

Chromatography is a very common method in chemical and biochemical analysis, which is used to separate components of a sample. There are many different types of chromatography, but all of them have the use of two phases in common. These phases are the stationary phase and the mobile phase. The stationary phase retains the sample while the mobile phase makes it flow. Thus, the affinity of the analyte to the stationary phase retains and determine its separate components.

3.1 TLC (Thin Layer Chromatography)

The TLC method is simple, fast and cheap. It is not as accurate as other methods such as HPLC (High performance liquid chromatography) or GC but allows separate lipids with its polarity. The sample runs for a plate, which can be silica or aluminum, the most common is silica gel G [2].

Samples can be applied manually or using a device developed by CAMAG laboratories called Linomat 5, which automates the process. The automatic version has many advantages, such as ensuring that the amount of sample is the same, the distance between spots is always the same and can play the same method as often as you like [3].

The next step in TLC is the development, which occurs in a glass chamber. We added the solvent, leaving clutter and put the plate, with the point where we wanted to stop the solvent previously marked. This process can be done automatically with the device called ADC2 [4]. We can control some conditions like humidity, saturation and you make sure that the solvent stops in the point that you choose. When development stops, there is an option of drying the plate before removing it.

The last step is the visualization of the TLC. Less destructive agents can be used or not, depends on the ultimate goal. Sometimes you need a UV device to view all bands [2]. The identification of the analytes is done with the R_F reference value. This value is obtained by dividing the distance the sample has run into the distance the solvent has run. Each analyte has its own R_F, thus it is easy to identify them.

This technique, HPTLC (High performance thin layer chromatography), is more accurate than the TLC, although it has similar principles. It is very useful for analyzing heavy and non volatile lipids [5]. The technique is based on two phases: the nonpolar or stationary phase, which is contained within a column. The other phase is polar phase or mobile phase, which separates the components according to their polarity.

1. parts 1, 2 and 3 are extracted from [1]

3.2 HPLC (High performance liquid chromatography) [6]

HPLC is improved classical liquid chromatography. It is based on the passage of a liquid mobile phase through a column which contains a fixed stationary phase. The analytes are separated as they interact with the mobile phase, whose concentration varies in time.

There are four types of liquid chromatography:

- Adsorption + distribution chromatography, where the stationary phase is an adsorbent and separation is based on various stages of adsorption and separation.
- Adsorption Chromatography, where the separation consists in distributing the solute between the mobile phase and the stationary phase.
- Ion-exchange chromatography, where the stationary phase contains ions that retain the analyte ions in the opposite direction of the sample flowing through the mobile phase.
- Molecular exclusion chromatography is based on using a porous stationary phase, which filters molecules selectively.

It can also be divided into two types: normal phase chromatography, where the stationary phase has polar points and produces specific interactions with the solute, and reverse phase chromatography, where the stationary phase is nonpolar and occurs hydrophobic interactions.

The chromatography on a HPLC consists of the following parts:

- Pump and device mixed eluents: The pump provides a constant flow of the mobile phase through the column. This has to bear high pressures and work steadily.
- Device injector: It is very important for the good functioning of chromatography. It has to introduce the sample with a band as close as possible. There are two types of injectors, which are using a syringe or valve.
- Pipes and connections: there is the need to minimize the length of the connections possible to avoid losses.
- Detector and recorder: The detector is capable of measuring a physical characteristic of the analyte. The detector used universally is the refractive index. It is possibly use ultraviolet light / visible, fluorescence and electrochemical detectors.
- Column: this is where the separation occurs, thus it is essential to choose a good column. The most used columns are filled, consisting of a tube filled with the chosen stationary phase. The characteristics of the column which affect the separation are the internal diameter, length, connections, and its particles size.

The choice of mobile phase is in accordance with literature and experience. For the four types of chromatography there are appropriate mobile phases.

The device detects the departure time of each molecule called retention time, and finally makes a chromatogram, where each peak represents the number of molecules that have eluted at a certain time.

4. Detection

4.1 MS (Mass Spectrometry)

The modern applied Mass Spectrometry methods are very different. I will focus on those used in the experiment.

Generally, the mass spectrometer operates in the following way: There are three components, the first one ionizes the sample positive (M^+) or negative (M^-). The following component separates the sample mass / charge and finally, the third component detects the analytes [7]. The three steps in analysis are called ion creator, ion separation and ion detector.

Table1. Different techniques for lipid analysis, separated for ionization, ion analysis and ion detector [7]

Ionization (Ion creation)	Ion Analysis (Ion separation)	Ion detector
Electron Impact (EI)	Quadrupole	Faraday cup
Fast Atom Bombardment (FABS)	Ion Trap	Electron multiplier
Electrospray (ESI)	Time of Flight (TOF)	Photomultiplier conversion dynode
Atmospheric Pressure Chemical Ionization (APCI)	Tandem MS (MS/MS)	
Atmospheric Pressure Photoionization (APPI)		
Matrix-Assisted Laser Desorption Ionization (MALDI)		

4.2 Ionization

4.2.1 ESI (Electrospray Ionisation)

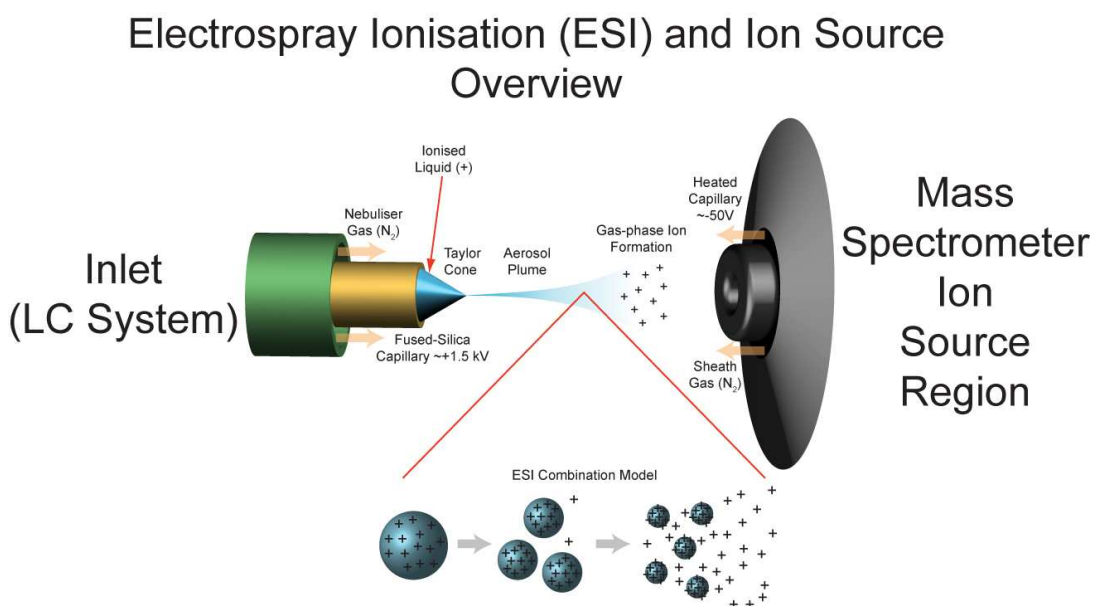


Figure1. Parts and uses of Electrospray Ionisation

LC-MS has two limitations [7]:

- a) Many molecules are unable to be ionized because they are not volatile.
- b) if the ionization is possible, it may be that m/z is too high or low, for this reason it can not be detected by the device.

ESI uses electricity to transfer ions from a solution to the gas phase. This aspect will be improved ESI-MS sensitivity.

The compound, which has been previously dissolved, goes through a highly charged capillary. The fluid leaves the capillary and forms an aerosol (small droplets) with a highly charge. Droplets are dispersed as the solvent evaporates and charges repel each other. When the whole solvent has evaporated, ions go through the mass spectrometer.

ESI has many advantages:

- The compound is ionized directly and allows the study.
- You can configure the mobile phase flow automatically.
- It produces multiple ions in one molecule, so that it can be analyzed with MS despite having very high weight.
- It has a higher accuracy.

4.2.2 MALDI (Matrix-assisted laser desorption/ionization)

The sample is applied to metal plate and when the solvent evaporates, sample are crystallized. Then the sample is attacked by pulse laser and is ionized to monocharged species. The area is heated and ions move from solid to gas phase. This ionized species are detected by an ion detector.

4.3 Analyser

4.3.1 TOF (Time of Flight)

The ions are accelerated in an electric field. Their mass is determined in a vacuum, using a precise measure of time than electrons leaving the source to hit the detector. The kinetic energy, which depends directly on the mass, determine the time of flight. It is used for non-target analysis as exact mass is obtained.

4.3.2 Single quadrupole

A quadrupole is an ion filter. It consists of a tube where two voltages run: DC (direct current) and RF (radio frequency). The combination of these voltages permits to filter molecules through their m/z (mass / charge). Ions whose m/z combine with DC and RF pass through the quadrupole with stable career, and the rest will be lost. It is used for target analysis and gives the nominal mass.

4.3.3 QqQ (Triple quadrupole)

The union of two quadrupole and a cell collision called triple quadrupole. This technique allows different types of analysis, depending on the objectives of the experiment.

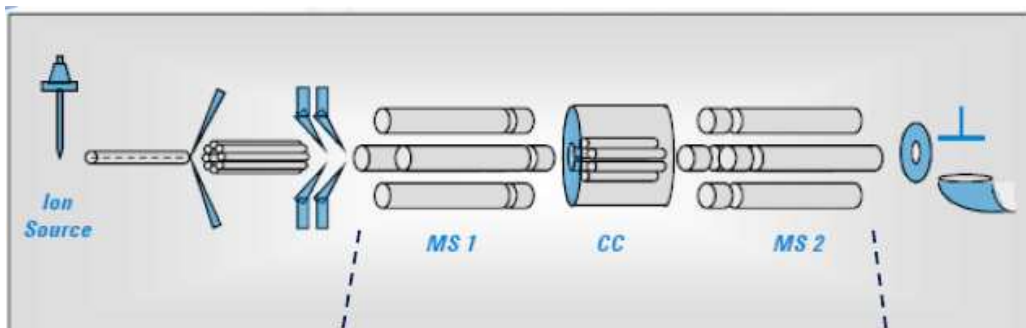


Figure2. A triple quadrupole. MS 1 is the first quadrupole, CC is the cell collision quadrupole, and MS2 is the third quadrupole.

This technique is focused for targeted and quantifications analysis.

The ion source enters ions into the device, that will do a general scan. In the first quadrupole is filtered ions that contain the m/z wished, that will pass into cell collision. There are fragmented into smaller ions, which will be filtered again in the third quadrupole. Finally the device expands the electrical signal and we will detect the amount of ions leaked.

Product ion scan and multiple reaction monitoring are quantitative techniques, and Precursor ion scan and neutral loss scan are qualitative techniques [1].

Product ion scan

It is a technique for finding the structure of introduced ions. Knowing beforehand the mass of the ion, it fragments into several smaller ions, and we detect the m/z of them. Before using this method, you need a pattern for calibration.

Selected reaction monitoring (SRM) / Multiple reaction monitoring (MRM)

This method is used for the detection of ions, because it increases sensitivity. The first quadrupole lets ions containing an m/z concrete, and after the collision, the second quadrupole filters the fragments ions that we want to analyze. It is necessary to know the m/z ions to leak.

Precursor ion scan

In this case, we know how an ion fragments and we use this technique to detect if this ion is present in the sample. For example, knowing that phosphatidylcholine is fragmented into ions 184 and 104, ordering only those two pieces were filters, so if there are in the same peak fragmentation it means that we have phosphatidylcholine.

Neutral loss scan

It is similar to the technique precursor ion, but in this case the fragment characteristic of the family of molecules that we search have not charge and it lost. The MS detects the initial molecule and after the fragments which have lost this neutral mass.

Table 1. It shows which quadrupole filter each scans according to the technique used. [1]

Scan mode	Q1	Q3
PIS	Fixed m/z	Scanning
PreIS	Scanning	Fixed m/z
NL	Scanning	Scanning
MRM	Fixed m/z	Fixed m/z

5. Types of phospholipids

Phospholipids may be defined as “Hydrophobic or amphipathic small molecules that may originated entirely or in part by carbanionbased condensation of thioester whose are fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids, and polyketides, and/or by carbocation-based condensations of isoprene units (prenol lipids and sterol lipids.” [8]

Phospholipids are the major component of cell membranes and liposomes. Phospholipids are an amphipathic molecules: it have a polar part (affinity for water, hydrophilic) and nonpolar part (non affinity for water, hydrophobic or lipophilic). There are many different types and consist of a glycerol, a phosphate group and two fatty acid chains.

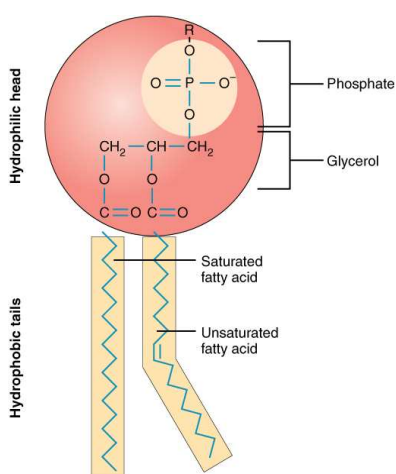
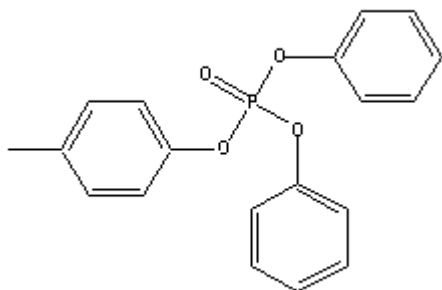


Figure3. Typical phospholipid. This is composted by hidophilic head, which contains phosphate and glycerol; and hydrofobic tails, whose contains saturated fatty acid or unsaturated fatty acid

The synthesis of phospholipids involves the esterification of alcohol on a phosphate of phosphatidic acid (1,2-diacylglycerol 3-phosphate). Alcohols that are added are usually

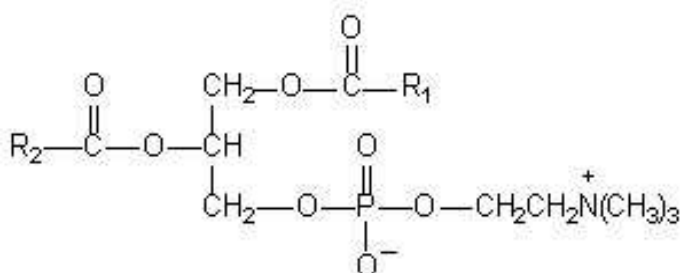
serine, ethanolamine and choline. In general, it can be synthesized in two ways: using a polar group activated with CDP for binding phosphate to phosphatidic acid, and using 1,2-diacylglycerol and non-activated polar group.



CDP from chemyq.com

The information about this phospholipids has found in [9] and its references.

5.1 Phosphatidylcholine (PC)



A phosphatidylcholine

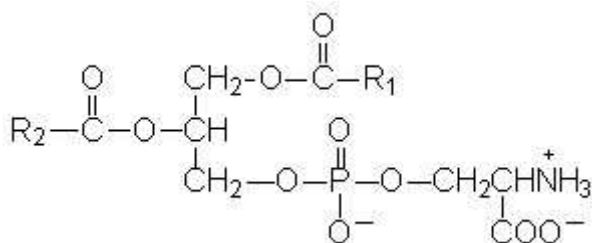
It is also known as lecithin. It contains palmitic acid and stearic acid on carbon 1, and carbon 2 contain oleic acid, linoleic or linolenic acids. There are three synthetic pathways [20]. The first pathway consists in activation of choline by phosphorylation and then coupling to CDP before binding to phosphatidic acid. The second way consists in adding choline to 1,2-diacylglycerol, which is activated by CDP. Finally, the third way consist in the conversion of phosphatidylethanolamine or phosphatidylserine to phosphatidylcholine [21]. Phosphatidylserine is decarboxilated and becomes PE, which suffering methylation reactions and becomes PC.

Because of its structure, phosphatidylcholine is spontaneously organized in lipid bilayers, which is a very important component of cell membranes. The unsaturations on carbon chains give fluidity [22].

The PC is also the most abundant phospholipid in plasma, because it is the only phospholipid essential for the packaging and secretion of lipoproteins. It is found in abundance in many HDL and influence in increasing the concentration of VLDL. Another important function is its role in cellular signaling, acting, according to some studies, in the cell nucleus [20].

It is the precursor of sphingomyelin, phosphatidic acid, lysofosfatidylcolina, phosphatidylserine and platelet-activating factor which is an important factor in signaling pathways.

5.2 Phosphatidylethanolamine (PE)

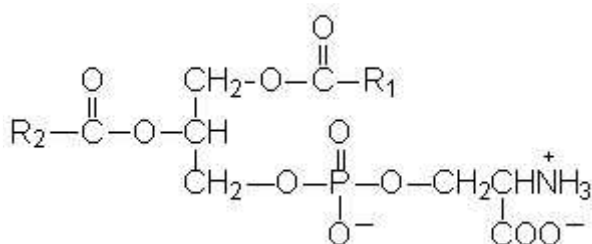


A Phosphatidylethanolamine

They are molecules that contain acid palmitate or stearic on carbon 1 and long unsaturated fatty acid on carbon 2. Phosphatidylethanolamine has two synthetic pathways [23]: the first one consists in ethanolamine phosphorylation, which is activated and, afterwards, coupled in CDP. Then, the ethanolamine is transmitted to phosphatidic acid and phosphatidylethanolamine is produced. The second way consists in PS decarboxylation.

Phosphatidylethanolamine is a N-acyl-phosphatidylethanolamine precursor [24], and it is a key component of cell membranes. It has a small head that gives the PE a cone shape. This head can form hydrogen bonds with several proteins. It forms inverted hexagonal phases which it helps maintain stability and modulates membrane curvature in lipid bilayers [23].

5.3 Phosphatidylserine (PS)



A Phosphatidylserine

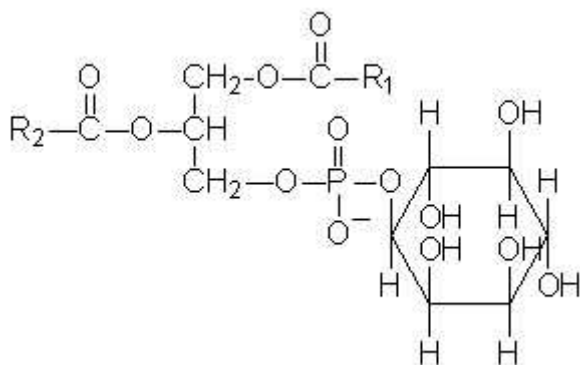
Phosphatidylserine can be synthesized from Phosphatidylethanolamine exchanging a serine with ethanolamine when Phosphatidylethanolamine is in the lipid bilayer. The reaction can also occur in reverse and get PE from Phosphatidylserine [25].

It is abundant in myelin from brain tissue, and it is mainly found in the plasma membrane and endosomes into cell. However, the concentration in the mitochondrial membrane is very low [26]. In plasma membrane the inside part has more PS as outside part [26].

Phosphatidylserine is an essential cofactor. It activates a several number of proteins, specially proteins which are involved in signal pathways. Besides, phosphatidylserine is

involved in the blood coagulation process in platelets and cell apoptosis. According to some studies it has health benefits such as preventing aging of the brain, improving memory and physical performance [27].

5.4 Phosphatidylinositol (PI)

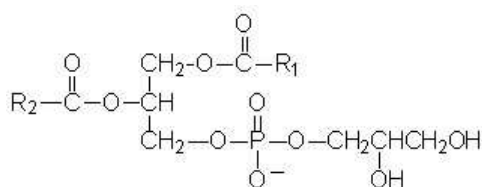


A Phosphatidylinositol

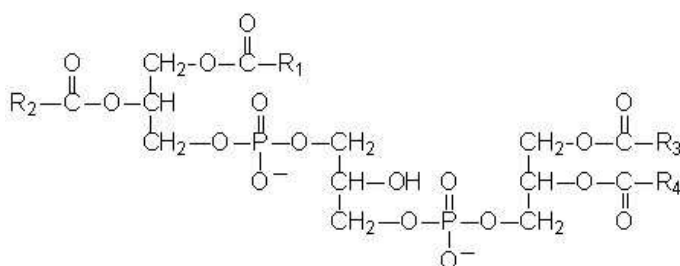
PI contains stearic acid on carbon 1 and arachidonic acid on carbon 2 . When inositol is phospholirated, it is called phosphoinositol. Phosphoinositol is a clue molecule for cell signaling, as it acts as a signal transducer [28].

It is synthesized by condensation of 1,2-diacylglycerol with CDP-activated by myoinositol [29]. Aferwards, PI is phosphorylates several times and eventually forms poliphosphoinositol, which is a phospholipid from membane. This poliphosphoinositol is critical for signalling involving growth and cell differentiation.

5.5 Phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG)



A phosphatidylglycerol (PG)



A Diphosphatidylglycerol (DPG)

PG is abundant in mitochondrial membranes and as components of pulmonary surfactant [30]. It is a precursor to the synthesis of cardiolipin and this is its main function. It is synthesized from DCP-diacylglycerol and glycerol-3-phosphate.

DPG molecules are very acidic. They are found in mitochondrial membranes and as components of pulmonary surfactant like phosphatidylglycerol. The most important class of DPG is cardiolipin, which are formed by condensation with CDP-diacylglycerol and PG.

This molecule, which is found in mitochondria, is crucial because it interacts with a large number of mitochondrial proteins. Specifically, participates in ATP production by activating enzymes. It is part of complex III and IV of the respiratory chain, and the ADP-ATP carrier [31].

It joins two kinase anchoring energy metabolism (mitochondrial creatine kinase and Nucleoside diphosphate kinase). This fact facilitates the transport of the inner membrane of the mitochondria.

It is implicated in the process of cell apoptosis, because it interacts with proteins that induce cell death, such as cytochrome c. The cardiolipin is an important cofactor for the translocation of cholesterol from the outer to the inner mitochondrial membrane. It is also involved in the packaging of mitochondrial proteins.

Materials and methods

We have divided the process into five parts, the extraction of phospholipids, the mobile phase for HPTLC chosen, the process used for the derivatization, the mobile phase chosen for liquid chromatography and its conditions and finally the pass from HPTLC plate to liquid analysis.

1. Lipid extraction

1.1 Extraction1: Bligh & Dyer method [10]

Put 50 µl of sample and add 950 µl of chloroform:methanol (1:2), vortex it during 30 seconds. After, add 320 µl of chloroform, vortex it during 1 minute, and add 320 µl of water, vortex another minute.

Centrifugate at 8000g during 10 minutes at 10 °C, remove the upper phase and collect the lower phase. Evaporate the lower phase with N₂. After evaporation redissolve the lower phase with 200 µl chloroform:methanol (2:1)

1.2 Extraction 2: phosphatidylcholines [11]

Put 50 µl of sample and add 320 µl of methanol, vortex during 30 seconds, add 630 µl of DCM (dichloromethane) and vortex during 1 minute, and introduce 200 µl of water. Then, vortex during 10 seconds and stay 10 minutes in a room temperature. Centrifugate at 8000g for 10 minutes at 10°C, Remove the upper phase and collect the lower phase

Evaporate the lower phase with N₂. Redissolve the lower phase with 200 µl of ethanol:chloroform (2:1).

1.3 Extraction 3: Extraction with MTBE [12]

Put 50 µl of sample and add 375 µl of methanol, vortex during 30 seconds. Add 1,25 ml of MTBE, and the mixture was incubated for an hour at room temperature in a shaker. Add 312 µl of MS-grade water and incubation during 10 minutes at room temperature. Centrifugate at 1000g during 10 minutes. Collect the upper phase and reextract the lower phase with 500 µl of mixture [MTBE:methanol:water (10:3:2,5 v/v/v). Combine both organic phases and dry in a vacuum centrifuge. Redissolve with 200 µl of chloroform/methanol/water (60:30:4,5)

1.4 Extraction 4: Liquid-liquid extraction using butanol [12]

Put 50 µl of sample and add 267 µl of phosphate buffer (30mM citric acid and 40 mM Na₂HPO₄). Vortex during 30 seconds, and add 666 µl of 1-butanol and 333 µl of water. Vortex during 15 seconds. Centrifuge at high speed for 10 minutes, collect the upper phase, dry with N₂. Finally, redissolve with 200 µl of ethanol

1.5 Extraction 5: Extraction with OSTRO [13]

These instructions are taken directly from the Waters Corporation.

Place the Ostro plate onto 2ml collection plate, and position in the vacuum manifold. Load 50 µl of the sample directly into the wells of the ostro plate using a micropipete. Add 400 µl of ethanol to the wells and thoroughly mix by aspirating the samples 10 x using a micropipete. After mixing it, apply 15" Hg of vacuum until the solvent drains completely. Repeat the steps 3 and 4. Remove the collection plate containing the flow through fractions from the vacuum device and replace with a fresh plate to collect the eluate fraction. Add 400 µl of elution solvent (4,5:4,5: 1 chloroform:methanol:triethylamine) to the wells. Repeat steps 7 and 8 and collect the eluted fraction. Dry the fraction with a nitrogen evaporator. Redissolve with 200 µl of chloroform:methanol 1:1 v/v

2. HPTLC - Mobile phases:

- 1- Chloroform:methanol:water:ammonia (60:34:4:2) [14]
- 2- Ammonium sulphate (20 mg) with petrol ether: ether: acetic acid (82:18:1) [5]
- 3- Chloroform: methanol: water (65:25:4) [15]
- 4- 0.4% ammonium sulphate and chloroform:methanol:acetic acid:acetone:water (40:25:7:4:2) [5]
- 6- *ethyl* acetate:2-propanol:chloroform:methanol:0.25% aqueous potassium chloride (abans methyl i 1-propanol) (25:25:25:10:9) + toluene:diethyl ether:ethanol:acetic acid (60:40:1:0,05) (65 mm and 80 mm) [5]
- 7- Hexane-ether (6:4) + chloroform:acetone:methanol (19:1:5) + chloroform:acetone:methanol:formic acid (85:10:5:0.6) (60 mm, 70 mm i 80 mm) [16]
- 8- chloroform:methanol:acetic acid:water (25 : 15 : 4 : 2) [15]

3. Derivatization [14]

Copper(II)sulfate: Dissolve 20 g of copper sulfate pentahydrate in 200 mL of methanol at less than 20 °C. Under cooling with ice add 8 mL of sulfuric acid 98 % and 8 mL of ortho-phosphoric acid 85%.

Immerse the plate into the reagent for 6 s. Dry the plate during 30 s with cold air and heat it at 140 °C for 30 min using a plate heater.

4. Lipid analysis

4.1 LC–MS conditions [17]

Separations ran for 30 min with mobile phase A and B consisting of 60:40 water:ACN in 10 mM ammonium formate and 0.1% formic acid and 90:10 IPA:ACN also with 10 mM ammonium formate and 0.1% formic acid, respectively. The gradient started at 32% B for 1.5 min; from 1.5 to 4 min increase to 45% B, from 4 to 5 min increase to 52% B, from 5 to 8 min to 58% B, from 8 to 11 min to 66% B, from 11 to 14 min to 70% B, from 14 to 18 min to 75% B, from 18 to 21 min to 97% B, during 21 to 25 min 97% B is maintained; from 25-30 min solvent B was decreased to 32% and then maintained. The column oven temperature was maintained at 45 C and the temperature of the autosampler was set to 4 C. The same LC conditions and buffers were used for all MS experiments with the flow rate was 260 μ L/min and the scan range was between m/z 120 and 2,000.

5. Scratching method [18]

The silica gel is scraped off the plate with a scalpel and extracted with chloroform/methanol/water (30:60:8, v/v/v). Vortex it during a minute. Afterward the sample is centrifuged and we put the supernatant.

Results

1. HPTLC results

These results correspond to application of the human plasma sample and the corresponding extraction in high resolution thin layer chromatography (HPTLC). We scanned the plates in UV after derivatization.

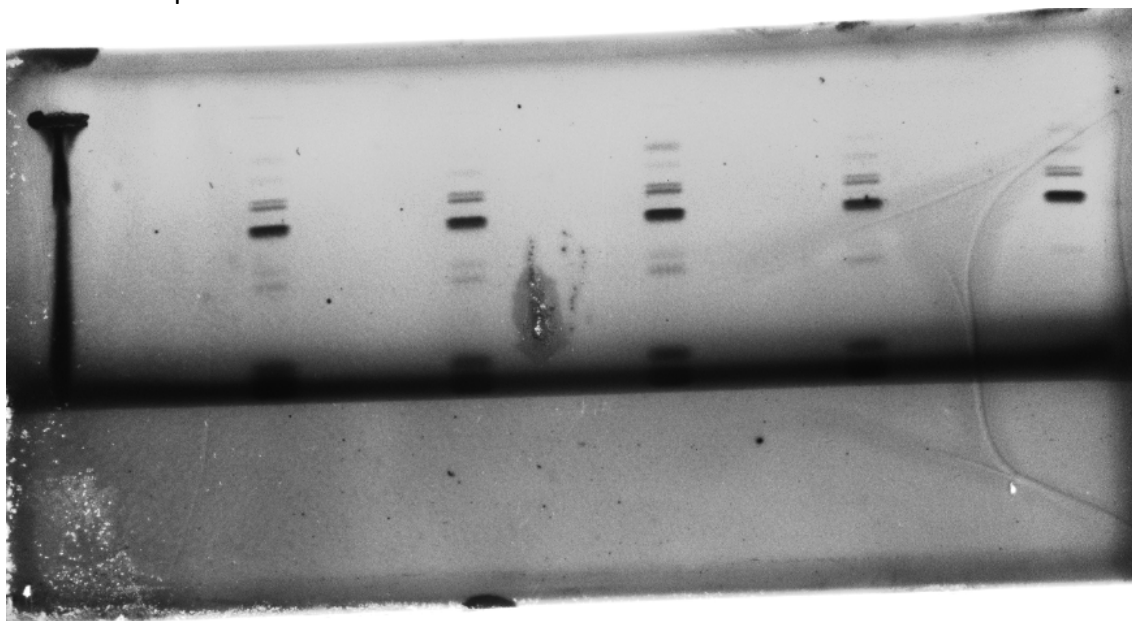


Figure 4. Human plasma and extractions 1, 2, 3, 4 and 5 running in mobile phase 1. We observed eight bands in carril corresponding to extrection 1, seven in carril corresponding to extrection 2, eight bands in carril corresponding to extrection 3, and extrection 4, and seven bans in carril corresponding to extrection 5. The most intense band was in the middle, in the same site in all of extractions, and corresponding to phosphatidylcholines [15].

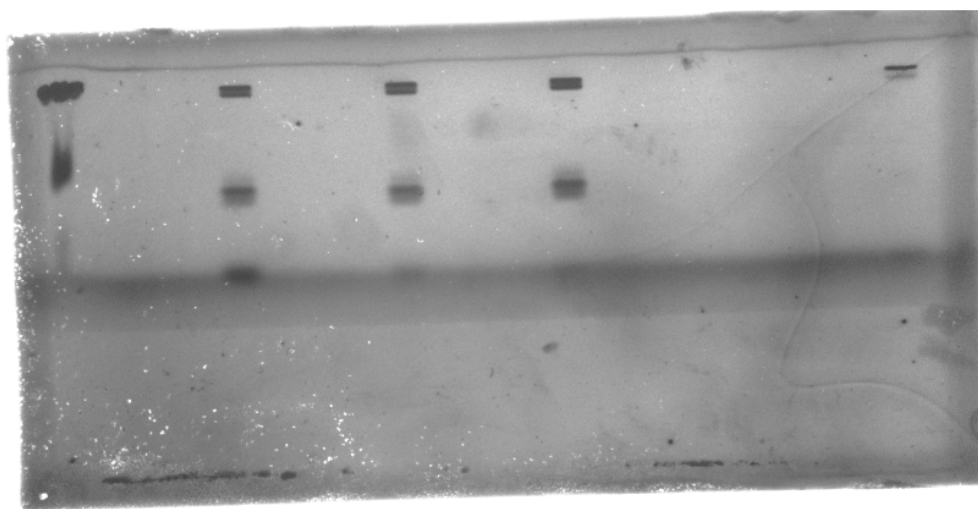


Figure 5 Human plasma and extractions 1, 2, 3, 4 and 5 running in mobile phase 2. We obtained four bands from extractions 1, 2 and 3, nothing from extraction 4, and only

one band from extraction 5. The most visible band corresponding to free fatty acids [15]

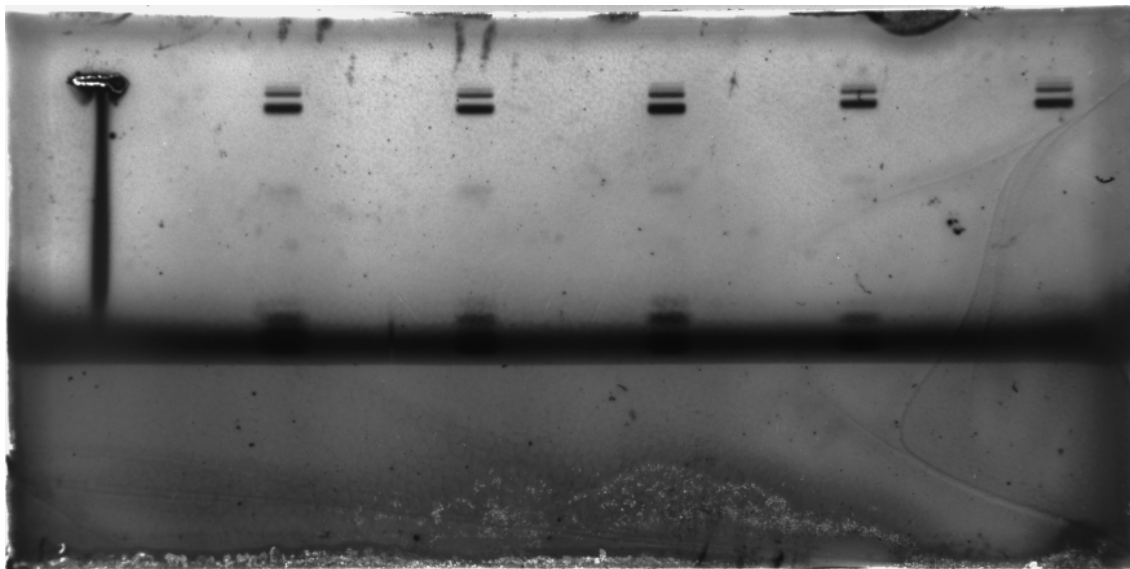


Figure 6. Human plasma and extractions 1, 2, 3, 4 and 5 running in mobile phase 3. There are four bands in all of extractions except in number 5. The most visible band corresponding to Lysophosphatidylcholine [15].

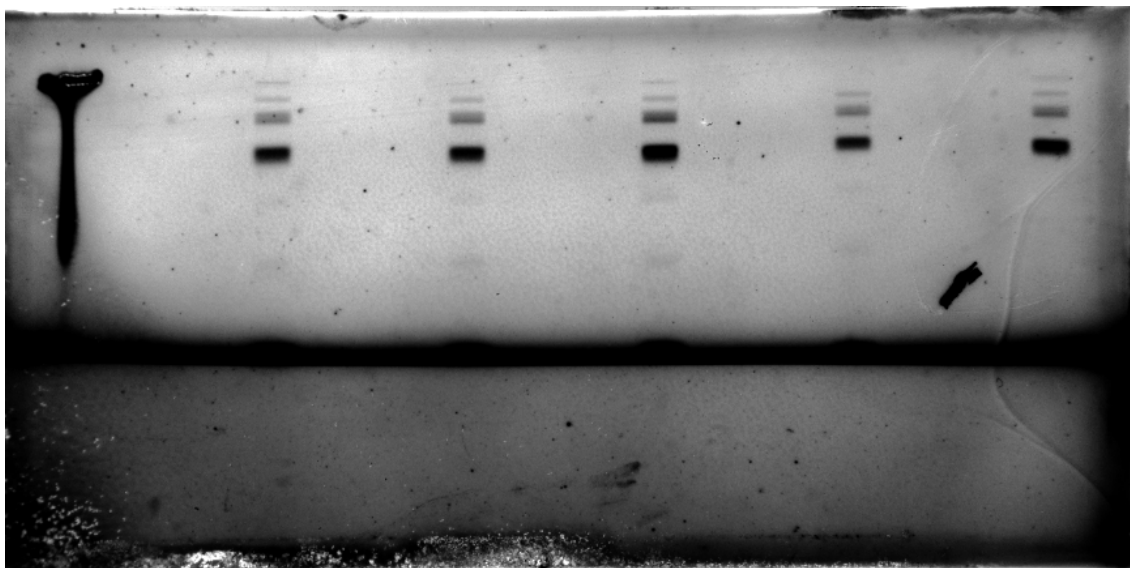


Figure 7. Plasma and extractions 1, 2, 3, 4 and 5 running in mobile phase 4. There are 5 bands in all of then except for extraction 5. The most visible band correspond to Phosphatidylcholine [15]

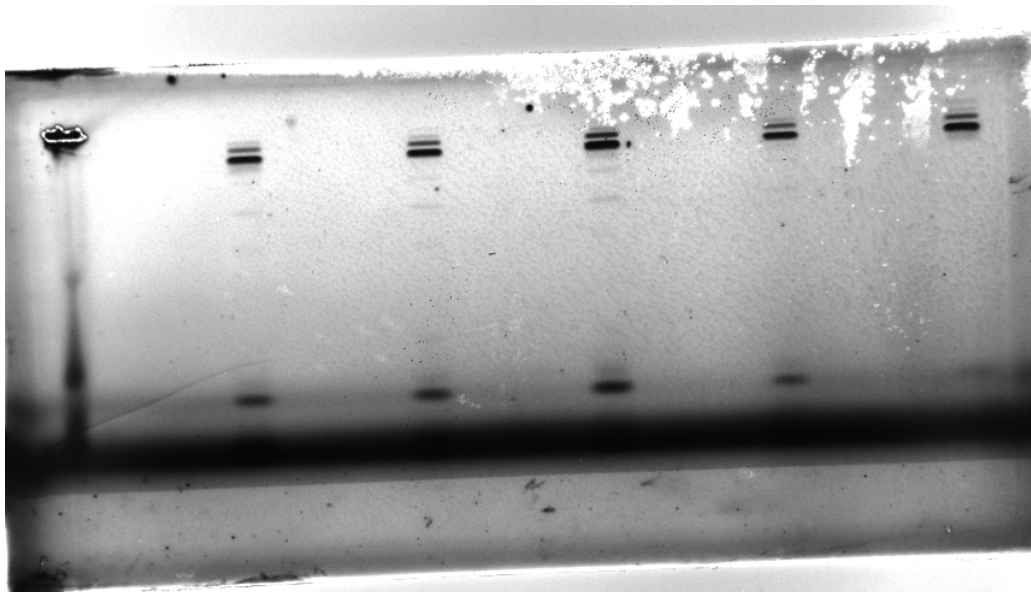


Figure 8. Plasma and extractions 1, 2, 3, 4 and 5 running in mobile phase 5. This mobile phase has composted by a mix with two disolvents. We obtained seven band in all of extractions except for extraction 5. In this case we only obtained four bands.

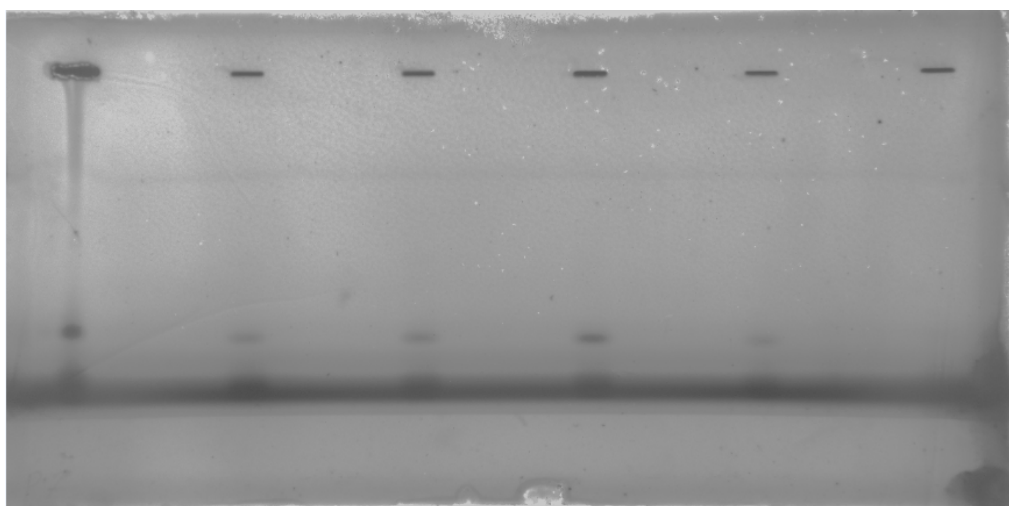


Figure 9. Plasma and extractions 1, 2, 3, 4 and 5 running in mobile phase 6. This mobile phase has composted by a mix with two disolvents. We only obtained a band from extractions 1, 2, 3 and 4. We didn't know the lipid that corresponding to this band.

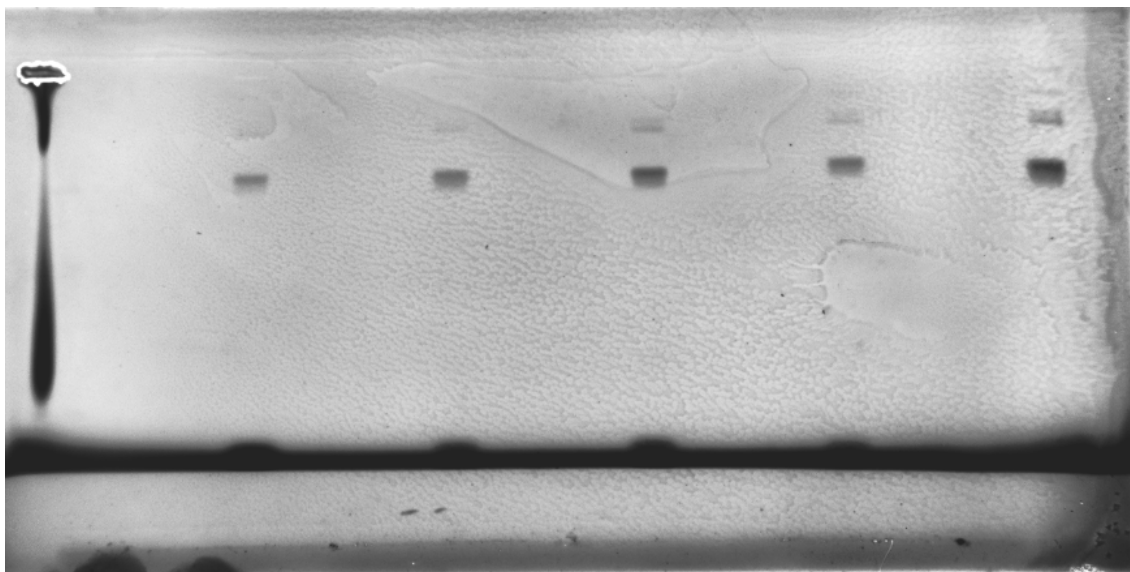


Figure 10. Plasma and extractions 1, 2, 3, 4 and 5 running in mobile phase 7. We obtained four bands in all of them. The most visible band corresponding to phosphatidylcholines [15]

In general, there are not significant differences between different statements in TLC. As it can be seen in some other extractions, there is no separation. In the mobile phase 2 the extraction number 4 is not separated. Besides, if it is examined closely, the extraction number five has less bands. It can be seen in all mobile phases except in the first one.

Many of the most intense bands of these chromatographic plates (figure 4, 7 and 10) correspond to phosphatidylcholine.

2. Q-TOF results

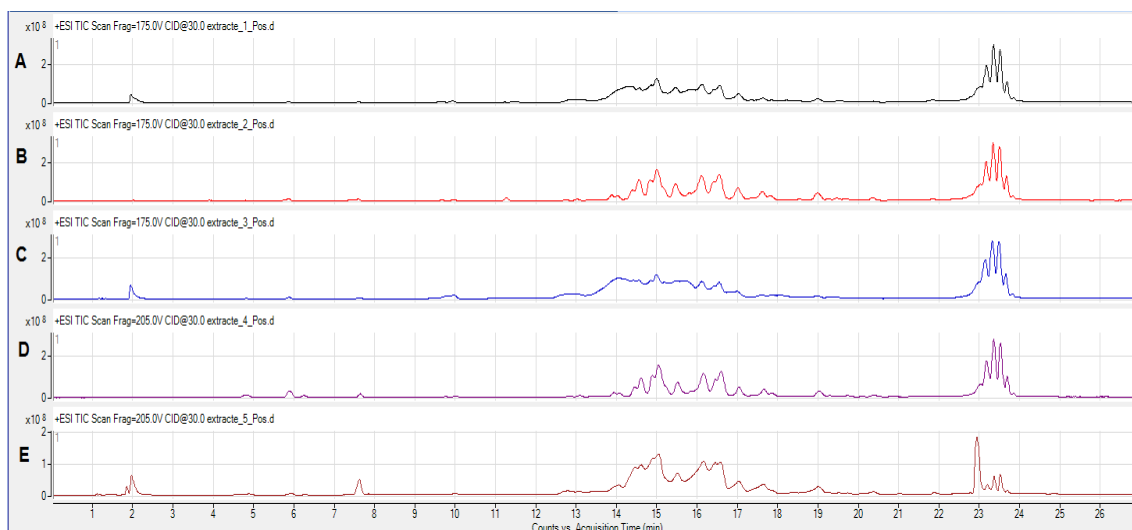


Figure 11. Analisis of five extractions in Q-TOF. A corresponding to extraction 1, B corresponding to extraction 2, C corresponding to extraction 3, D corresponding to extraccion 4 and E corresponding to extraccion 5.

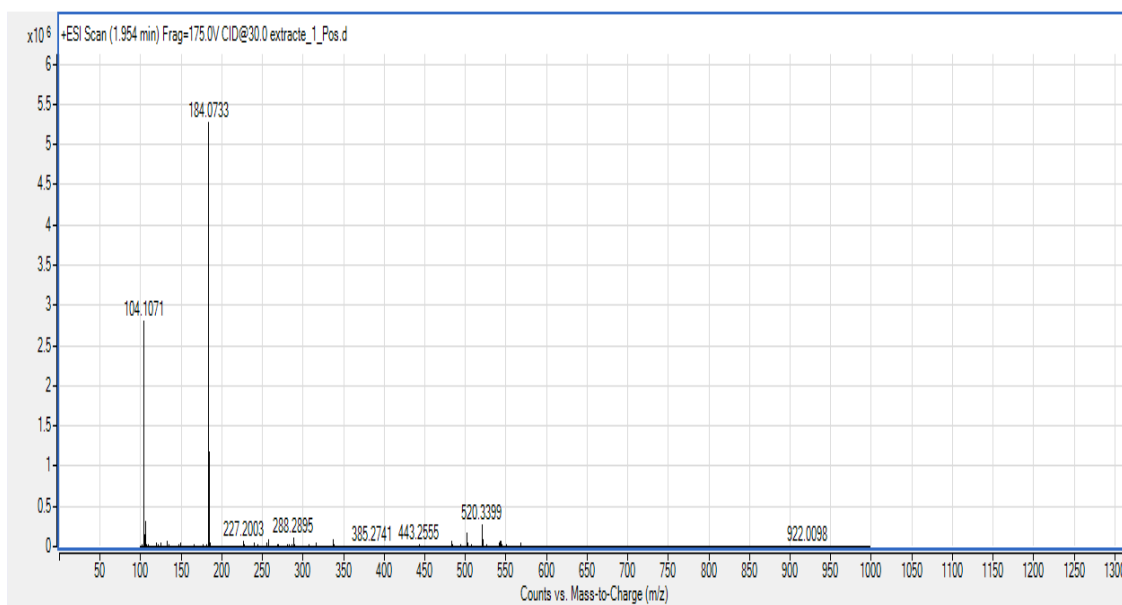


Figure 12. An example of Q-TOF Mass spectrum. We found a phosphatidylcholine with 520.3399 m/z, corresponding to $C_{26}H_{51}NO_7P$ [8].

We analyzed the five made statements by HPLC coupled to Q-TOF in positive using the mobile phase for HPLC explained in materials and methods. Regarding the level of phospholipids found, are the same in all five extracts. We found 21 different phosphatidylcholines, which correspond to different peaks [8].

At the same time we analyzed four bands taken from the plates 2, 3 and 5 using the method described above [18]. No phospholipid was found, because we did not find any reference in the lipid maps website [8]. Besides, we put a sample from a plate 2 which RF corresponding to free fatty acids and not to phosphatidylcholine or other phospholipid. Therefore, the peaks shown in the graphs of chromatograms are possibly the remains of silice.

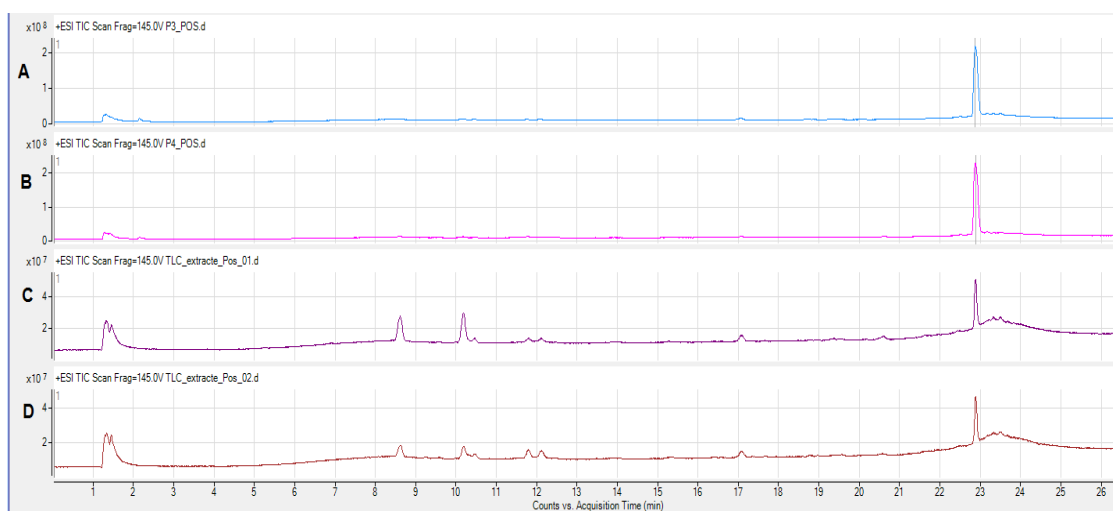


Figure 13. A Q-TOF analysis. A is extracted sample from plate 3 corresponding to the band of phosphatidylcholine, B is extracted sample from plate 2 corresponding to the

band of free fatty acids, C is extracted sample from plate 1 corresponding to the band of phosphatidylcholine, D is extracted sample from plate 1 corresponding to the band of phosphatidylethanolamides.

3. QqQ results

We proceeded to do the assay of our TLC samples in QqQ, because it has a higher sensitivity [7]. This was analyzed with the method "precursor ion", setting the following ions (m/z) in positive: 184 and 104 (phosphatidylcholine) and 148 (phosphatidylethanolamides) [15][19]. We obtained the following chromatogram for phosphatidylcholine, while in the analysis for phosphatidylethanolamides we found nothing.

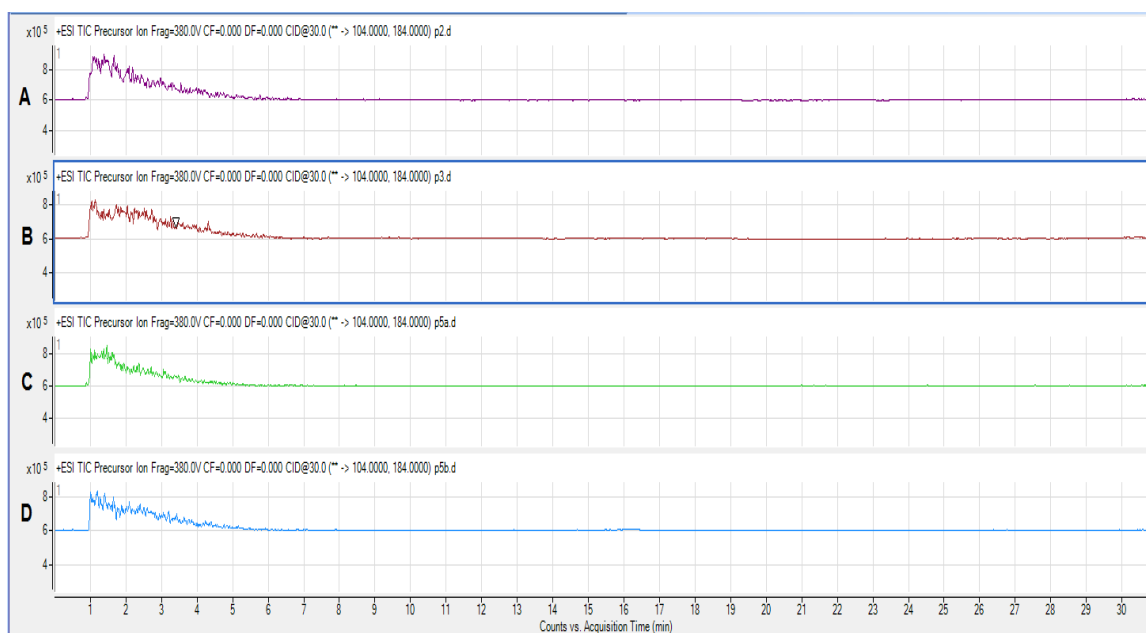


Figure 14. QqQ analysis. Precursor ion (m/z) 104 and 184 (phosphatidylcholine) A corresponding to extracted sample from plate 3 from the side of phosphatidylcholine, B corresponding to extracted sample from plate 2 to extraction from the side of free fatty acids, C corresponding to extracted sample from plate 1 to extraction from the side of phosphatidylcholine, D corresponding to extracted sample from plate 1 to extraction from the side of phosphatidylethanolamides.

In the second analysis was applied to vary the concentration of the plate, increasing it tenfold. We used extraction 1 and mobile phase number 4 because we have their exact R_f values.

We scratched the bands corresponding to phosphatidylcholine and phosphoglycerol and analyzed with the technique precursor ion (m/z) 184 and 104 and technology neutral loss with 189 m/z. We also evaluated the extract without TLC for comparative.

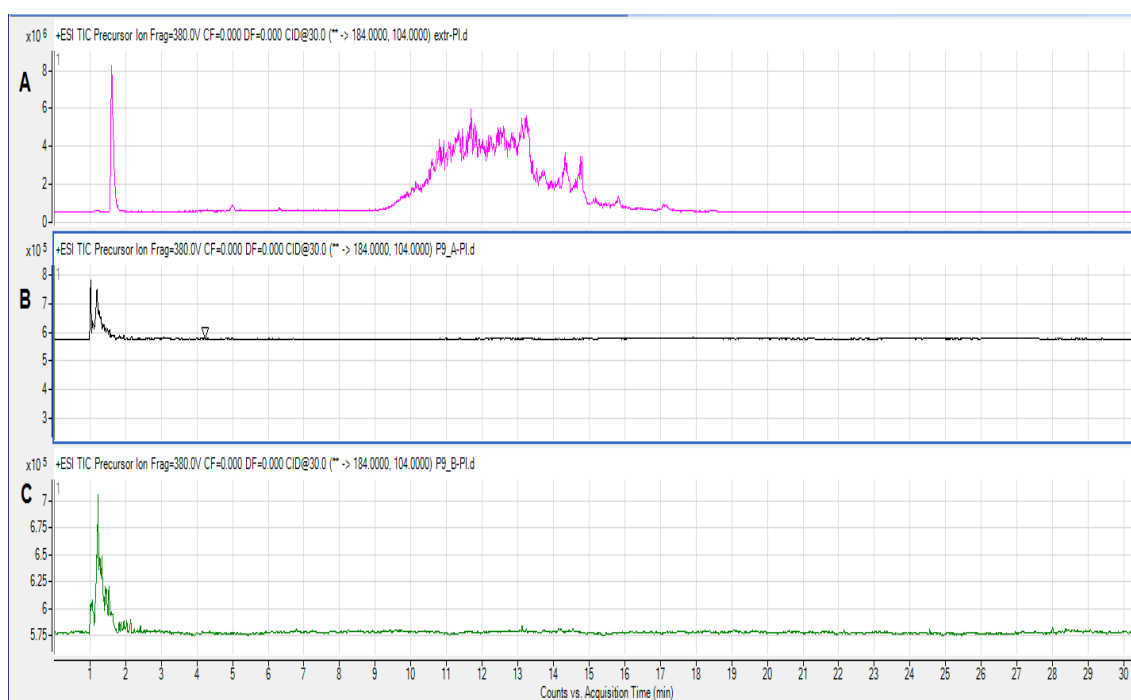


Figure 15. QqQ analysis with precursor ion m/z 104 and 184 (phosphatidylcholine) A corresponding to extraction without TLC, B corresponding to extraction from the side of phosphatidylcholine, C corresponding to extraction from the side phosphatidylglycerol

We found a clear peak in the analysis of extraction corresponding to phosphatidylcholine C38H71NO8P and its isomers [8]. There is nothing relevant extracts of the bands, either in the neutral loss analysis.

Repeated plate number 4 without derivatization end. Extract from the plate was taken as the reference plate number 4, because without derivatization we could not see the bands. We analysed precursor ion (m/z) positive for phosphatidylcholine (184, 104) and phosphatidylserine (192) and negative for phosphatidylinositol (241), as it took the bands corresponding to these phospholipids.

Table 2. R_f values of phospholipids separated in mobile phase 4. We scratch the bands corresponding to Phosphatidylserine, Phosphatidylinositol, Phosphatidylcholine and Lysophosphatidylcholine. [15]

Compound	$R_f \times 100$
Phosphatidylethanolamide	40
Phosphatidylserine	13
Phosphatidylinositol	13
Phosphatidylcholine	20
Lysophosphatidylcholine	6

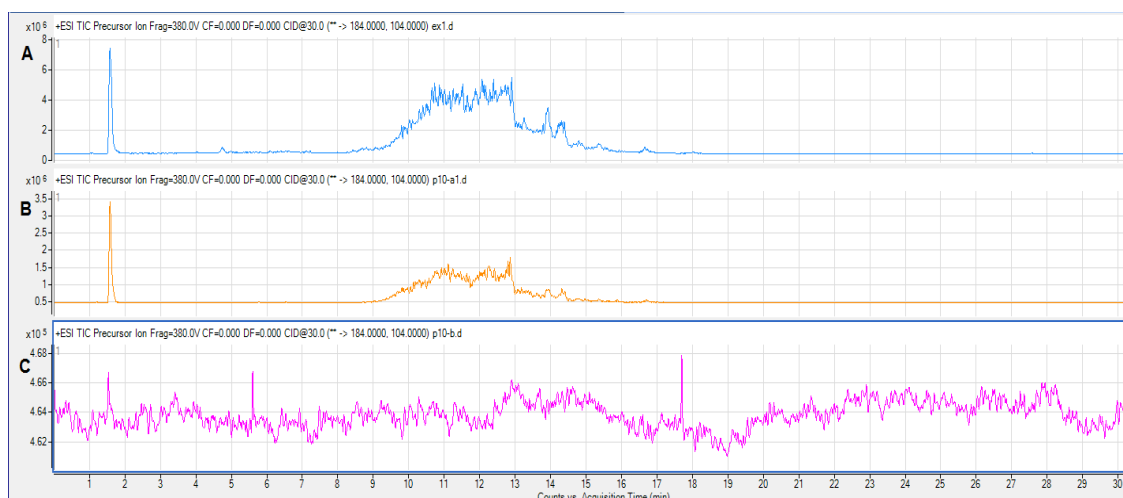


Figure 16. QqQ results with precursor ion (m/z) 104 and 184 (phosphatidylcholine) in positive. A corresponding to extraction without TLC, B corresponding to extraction from the side of phosphatidylcholine, phosphatidylserine and phosphatidylinositol, C corresponding to extraction from the side of no-separated lipids.

We found the same three phosphatidylcholine in extraction without TLC and the extraction of the plate, corresponding to [C₂₈H₅₅NO₇P](#), [C₂₆H₅₃NO₇P](#) and [C₄₂H₈₅NO₈P](#) [8]. We did not find anything on the analysis of the phosphatidylserine or phosphatidylinositol.

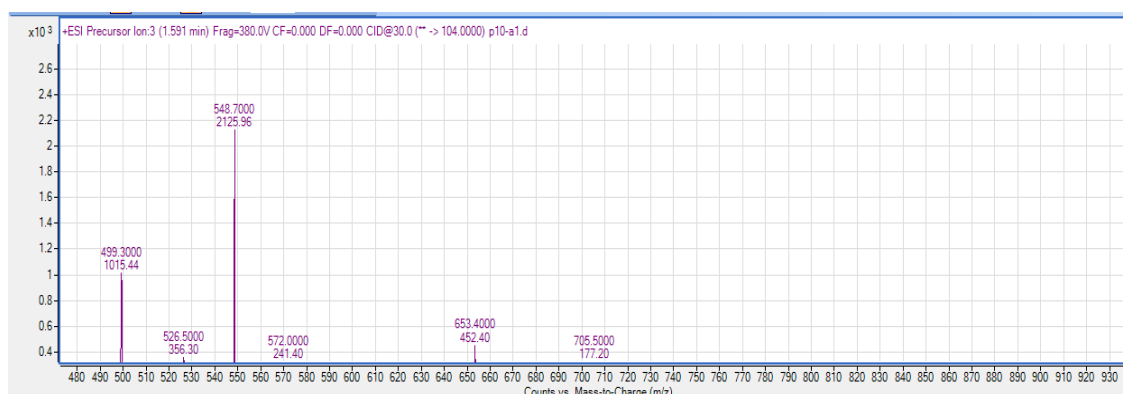


Figure 17. An example of QqQ Mass spectrum. We found a phosphatidylcholine with 548,700 m/z , corresponding to [C₂₈H₅₅NO₇P](#) [8].

Discussion

It is expected that there are not differences between the various extractions, as with all, except the fifth method, was used with a separation of two phases, the organic and the aqueous phase. We only varied the ratios of the same solvents (methanol, chloroform, water, ethanol...).

The extraction 5 method was taken from commercial house called Waters Corporation, allowing only the extraction of phospholipids. We could see that this extraction was valid because with the mobile phase 1, which separates only phospholipids, the same bands were obtained in all spots.

As explained in the introduction, the mobile phase separate the lipids for their polarity. There are three solvents used in different ratios: methanol, chloroform and water. There is no way to know the ideal mobile phase, but must be based on the literature and experience. In addition, results may vary depending on the environment and the technique used (TLC or HPTLC). For this reason we tried several mobile phases.

Mobile phases were chosen for further analysis: the mobile phase 1, 2, 3, 7 and 4. The phases 1, 2 and 3 were chosen by a non-target analysis (Q-TOF), because we did not know the exact RF values. We knew RF values from the mobile phases 7 and 4, for this reason we used them in a target analysis (QqQ).

The chromatogram resulting from the Q-TOF analysis reveals few differences between extraction and reinforces what has already been seen on HPTLC: it has taken almost the same with all methods.

In the case of extracts from the bands of the plate, the absence of lipids could be due to several reasons: they had not applied enough sample HPTLC plate, the method of derivatization was totally destructive or the step from plate to HPLC and Q-TOF analyzer was incorrect.

The analysis of phosphatidylcholine to search using the technique of extraction QQQ plates 1, 2 and 3 was irregular, while not found any results corresponding to fosfatidiltanolamides. In this case the peaks did not match any known phosphatidylcholine, besides its shape was very irregular.

After increasing the concentration and performed the QqQ analysis, we did not see notable peaks from extractions from HPTLC plates, and its peacks mismatch with the peacks from extraction without TLC. Thus increasing the possibility that the high derivatization involves destruction of the sample and the rest of phospholipids are not detected by QqQ.

There is no remarkable peak on analysis by neutral loss corresponding to phosphatidylglycerol [8], so you can determine that there is not phosphatidylglycerol or there is in very low concentration.

In this case we can see that it has been extracted phosphatidylcholine correctly, because the chromatogram from TLC bands is very similar to the chromatogram from extraction without TLC. We can also see that there is not any phosphatidylcholina that has not been separated since this ones do not appear in extract C (figure 16), which includes not separated compounds. Phosphatidylserine and phosphatidylinositol were not detected.

In total, we only found phosphatidylcholines in Q-TOF and QqQ. This phospholipid is the most common phospholipid in the organism and it is easier to detect. Other

phospholipids such as phosphatidylserines or phosphatidylethanolamides are found in a very low concentration and they may be lost during the the plasma extraction. The work could also be followed using MALDI ionization technique, but it was not possible due to the incompatibility HPTLC plates available in the center and MALDI device.

Conclusions

We have developed HPTLC technique, which was one of the objectives of the work, and found that it works properly varying work conditions. We found that the prior HPTLC separation is not required for the correct identification of phosphatidylcholine with MS (Q-TOF and QqQ). We did not observe a higher number of phospholipids, which was our goal. Therefore, to accurately determine the phospholipids of a sample is better to use the techniques HPLC coupled to Q-TOF (non-target analysis) or HPLC coupled to QqQ (target analysis). However, HPTLC technique separates phospholipids efficiently even in low concentrations (figures 4-7), so it is a technique that can be used in a qualitative way to get fast visually results.

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