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Methods of extraction and thin-layer chromatography determination of phospholipids in biological samples

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Abstract

Phospholipids (PLs) are a class of compounds essential for living organisms, including human beings. This paper presents a brief review concerning the extraction as well as thin-layer chromatographic separation and determination of phospholipids. After a short introduction regarding phospholipids and their specific characteristics, the extraction techniques will be discussed. Then, phospholipid separation and system detection for thin-layer chromatography (TLC) will be presented. The focus will be on phospholipids that are most abundant in biological systems; that is why chosen examples of PLs determination in biological samples will be revealed as well as general PL determination schemes. In the Summary section, there will also be a short discussion about advantages and disadvantages of PL determination with the use of TLC.

Keywords: biological samples; extraction of phospholipids; phospholipids; TLC detection of phospholipids.

Introduction

Phospholipids (PLs) are a class of lipids consisting of an alcohol backbone-glycerol (a 3-carbon chain molecule) or sphingosine (an 18-carbon amino alcohol with an unsaturated hydrocarbon chain) – combined with fatty acids esterified at the *sn-1* (first carbon of glycerol chain) and *sn-2* (second carbon of glycerol chain) positions (Alberts et al. 2004, Rastogi 2005). The fatty acid molecules may be from 14 to 22 carbon atoms long and have from zero to six double bonds (Schiller et al. 2004). The phosphate group at the *sn-3* position of glycerol chain is attached to a PL's polar head group, for example: choline, serine, etc. The general scheme of PL structure is shown in Figure 1. Depending on the type of the X substituent (the PL polar head), one can distinguish choline-containing phosphatidylcholine (PC), serine-containing phosphatidylserine (PS), glycerol-containing phosphatidylglycerol (PG), ethanolamine-containing phosphatidylethanolamine (PE), and inositol-containing phosphatidylinositol (PI).

PLs constitute 60% of the lipid mass of eukaryotic cell membranes (Han and Gross 2004). Their distribution in the cell membrane is asymmetrical; the outer layer contains mainly PC, while the inner side is mostly composed of PE and PS (Bevers et al. 1998). These compounds are precursors of arachidonic acid, which can be enzymatically decomposed into leukotrienes, prostaglandins, and thromboxanes – important modulators of inflammation. PLs are also precursors of platelet-activating factor and inositol triphosphate (Radziwon et al. 2004, Zemski Berry and Murphy 2004). PLs are the building blocks of biological membranes and tissues and are also frequently found in body fluids. Disturbances in PL metabolism can cause serious illnesses such as Alzheimer's disease, multiple sclerosis, or Niemann-Pick disease. Laboratories that perform prenatal diagnostics use thin-layer chromatography (TLC) for determining PG or the lecithin/sphingomyelin ratio to assess lung maturity, which provides very valuable and useful information for the clinician (Sapa 2006). PLs, especially PS, are crucial to the proper functioning of nerve cells as well as maintaining and restoring the activity of the receptors and proteins associated with cell membranes. They are also essential in the processes of cell differentiation, proliferation, and regeneration. Treatment with PS leads to an improvement of storage and retrieval of information. PLs improve cognitive abilities and the reception (perception) of information, "preprocessing" it in so-called short-term memory (Bodera 2009). The last but not the least, PLs may serve as biomarkers in the treatment of Alzheimer's disease (Kosicek et al. 2010).

Owing to the specific characteristics of individual compounds, it is important to develop quick, cheap, and simple methods for their extraction and detection. Therefore, this work presents the currently used methods of PL extraction and determination by TLC. TLC is the method of choice because it is one of the few techniques that can ensure fast quantitative interpretation; it is cheap, simple, and very insusceptible to contamination, making it competitive against more complex methods such as high-performance liquid chromatography (HPLC), gas chromatography (GC), or mass spectrometry (MS).

Extraction

The first step in the determination of PLs is their extraction from biological samples. It is an essential element of the process, as it prevents the impact of other components of the sample, i.e., proteins or sugars, which may cause interference or noise in the resulting chromatograms. The literature describes several methods used to extract PLs from samples, mainly based on liquid-liquid extraction (LLE) and solid phase extraction (SPE).

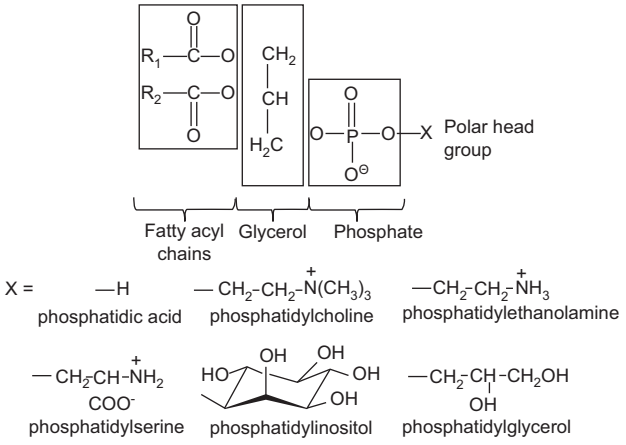

Figure 1 The general scheme of PL structure.

The literature also provides some information about PL determination by TLC without extraction (Cherayil and Scaria 1970).

Liquid-liquid extraction

LLE is performed by using two immiscible liquids that form two phases. Typically, one phase is an organic hydrophobic solvent, and the other one – a hydrophilic liquid. Owing to the highly lipophilic nature of LPs, they are soluble in organic solvents. The type of solvent is specifically selected and depends on the type of fatty acids present in the molecule and also on the nature of the lipids themselves.

PLs, due to the complexity of their structure, require the use of a polar organic solvent such as methanol or ethanol. Folch proposed the most popular type of LLE for PLs, which requires the use of a mixture of chloroform and methanol (2:1, v/v) (Folch et al. 1957). Subsequent modifications of this method were presented by Bligh and Dyer, who added water as a third solvent to the extraction mixture described above (Bligh and Dyer 1959). This made it possible to complete PL extraction in a single analytical step. The primary advantage of the Bligh and Dyer method is a reduction in the solvent/sample ratio (1 part sample to 3 parts 1:2 chloroform/methanol followed by 1 or 2 parts chloroform). In contrast, the Folch method employs a ratio of 1 part sample to 20 parts 2:1 chloroform/methanol, followed by several washings of the crude extract. A widely discussed comparison of the Bligh and Dyer and Folch methods for total lipid determination was made by Iverson and coworkers (Iverson et al. 2001). Generally, Folch's method is applied to biological tissue samples and Bligh-Dyer's method to biological fluid samples (Schiller et al. 2004), but not always. Avalli and Contarini used Folch's method for milk, butter, and buttermilk samples (Avalli and Contarini 2005); also, Donato et al. used it for the extraction of PLs from cow's and donkey's milk samples (Donato et al. 2011).

Another common method for PL extraction, developed by Radin, employs a mixture of hexane and isopropanol (3:2, v/v) (Radin 1981). This method has an important advantage of being less toxic than the methods described above. However, it is not so efficient, as Gunnlaugsdottir and also Tanamati found it to extract a smaller amount of polar lipids from meat samples with medium and high fat content than Folch's or Bligh-Dyer's methods (Gunnlaugsdottir et al. 1993, Tanamati et al. 2005).

The extraction of acidic PLs such as PS or PI is performed by using a mixture consisting of chloroform, methanol, and 12 mol/dm^3 hydrochloric acid (2:4:0.1 v/v/v) (Honeyman et al. 1983, Leßig and Fuchs 2009). However, also this method has a major drawback, namely, it causes a complete hydrolysis of plasmalogens (Leßig and Fuchs 2009), which are a type of ether PLs characterized by the presence of a vinyl ether linkage at the *sn-1* position and an ester linkage at the *sn-2* position (Nagan and Zoeller 2001, Gorgas et al. 2006, Moser et al. 2011).

Solid phase extraction

LLE methods are very effective, but require the use of organic solvents that may be hazardous to human health and life. They are also time-consuming. An alternative to them could be SPE using small disposable columns filled with suitable packing material. The columns are easy to use and utilize. Depending on the analyte, it is important to select appropriate columns and also an adequate eluting system. In extracting PLs, the packing material for columns is usually silica gel, unmodified or modified with cyanopropyl, aminopropyl, or dihydroxypropoxypropyl groups (Ruiz-Gutierrez and Perez-Camino 2000). The liquid phase is usually an organic solvent such as methanol, chloroform, hexane, or a mixture thereof, which is used for the elution of PLs from the columns (Kim and Salem 1990).

Typical steps in SPE include: (1) column conditioning to allow interaction between the solid phase and the sample, (2) sample application, (3) column washing to remove substances that interact weakly with the resin, and (4) elution of the analyte to remove the desired material bound to the solid phase (Flurkey 2005).

SPE is also a widespread technique for separation of PL classes (Christie 1982). This technique has been widely used, due to its easy and fast sample preparation. Pietsch and Reinhard (1993) developed a SPE method for the separation of individual PL classes (PC, PE, PS, and PI) using single aminopropyl SPE cartridges and standards of PLs. However, this procedure was not suitable for the separation of a mixture of PS and phosphatidic acid (PA) because they coeluted.

Later, Banni et al. (2001) separated liver PL classes according to the method described by Pietsch and Reinhard (1993) with minor modifications. It can be concluded that the modifications proposed for the Banni et al. (2001) SPE method for separation of PL classes allow the correct elution of PC, PE, PS, and PI using both standards of PLs and muscle samples. The effectiveness of the Banni et al. method studied to separate PL classes was visually checked by Perez-Palacios et al. (2007) and is shown in Figure 2.

A comprehensive review of the different procedures and methodologies for SPE used in lipid component separation

Figure 2 TLC plate of eluted PL fractions from the longissimus dorsi muscle of rats obtained following the Banni et al. (2001) modified method by Perez-Palacios et al. Reprinted from Food Chemistry, 102, Perez-Palacios T., Ruiz J., Antequera T., Improvement of a solid phase extraction method for separation of animal muscle phospholipid classes", 875–879, 2007, with permission from Elsevier (License number 2806480891666).

was made by Ruiz-Gutierrez and Perez-Camino (2000). Authors presented the analytical systematic method designed to separate groups of compounds and also specific components by using a combination of chromatographic supports and solvents. The review is divided into three parts, edible fats and oils, fatty foods, and biological samples in which the most common SPE techniques are presented.

Compared to LLE, the consumption of organic solvents in SPE is much smaller, and it is both a fast and reliable method of extraction. However, most publications still report the use of LLE as described above. This may be due to the fact that SPE is more susceptible to sample size. If too large a sample is added to an SPE column, it becomes saturated and reduces the recovery of PLs.

PL separation by TLC

TLC is one of the oldest forms of chromatography used for PL detection and separation; it is still successfully used today.

The most common stationary phase used in the separation of PLs in normal phase chromatography is silica gel (Steele and Banks 1994), and there are several modifications of this type of stationary phase available. Vaden et al. presented a quite simple method for separating complex lipid mixtures. They investigated the use of boric acid for PL separation from yeast and acidic lipids. They showed that the concentration of boric acid had a significant impact on the separation of PG and PE, with 1.8% boric acid being the optimum concentration (Vaden et al. 2005).

There are several ways of chemical modification of silica gel, which can improve PL partition, such as cover plates with magnesium silicate or oxalate (Singh and Jiang 1995), the addition of ethylene-diamine-tetraacetic acid (EDTA) or ammonium sulfate to improve the separation of acidic PLs (e.g., PS and PI) (Kaulen 1972, Allan et al. 1982, Ando and Saito 1987), and the use of boric acid or silver nitrate (Myher and Kuksis 1995). An extensive review of PS separation and determination techniques was written by Abidi (1998).

Another method of PL separation is based on the use of 0.01 M oxalic acid for the impregnation of Chromarods-SII plates (De Schrijver and Vermeulen 1991). Also, the use of ammonium sulfate as a modifier of the stationary phase has been reported to improve the separation of PI and PS (Wang and Gustafson 1992).

Nowadays, the rapid progress of science has contributed to the development of new stationary phases, such as Celite™ (Supelco Inc., PA, USA), cellulose powder, ion exchange cellulose, starch, polyamides, and Sephadex™ (Supelco Inc., Philadelphia, PA, USA), which may prove useful in the separation and determination of PLs (Fuchs 2011).

The mobile phase used for PL determination consists of solvents with different polarities. Most often, a mixture of chloroform, methanol, and water combined in different proportions is applied, although triethylamine, ethanol, hexane, and isopropanol may be used.

Leray et al. first described a typical mobile phase that allows an efficient separation of all the main PL classes for their quantification. It consisted of chloroform, ethanol, water, and triethylamine mixed in a volume ratio 30:35:7:35 and allowed a complete separation of cardiolipin (CL), lysophosphatidylcholine (LPC), PA, PC, PE, PI, PS, sphingomyelin (SM), and is shown in Figure 3.

Figure 3 TLC separation of PL classes according to Leray et al. Reprinted from Journal of Chromatography B: Biomedical Sciences and Applications, 420, Leray C., Pelletier X., Hemmendinger S., Cazenave J-P., "Thin-layer chromatography of human platelet phospholipids with fatty acid analysis", 411–416, 1987, with permission from Elsevier (License number 2806570317332).

Although this method was originally described for TLC plates impregnated with H_3BO_3, common TLC plates may also be used without a meaningful loss of quality. To receive better separation of the PLs with the lowest R_f, a second run in the same solvent following a rapid drying is recommended; also, because the solvent mixture is poorly volatile, the solution can be used for several runs for at least 2 days (Leray et al. 1987).

The use of mixture consisted of chloroform, methanol, and 2 N ammonia as mobile phase mixed in a volume ratio 65:24:4 allowed not only a complete separation of PC, PE, PI, PS on a single chromatoplate but also their quantitative determination (Vecchini et al. 1995).

Ruiz and Ochoa presented a new combination of solvent systems for the resolution of the major PLs and neutral lipids in one dimension on one chromatoplate. The complete lipid analysis of 24 samples took c.a. 90 min; spotted plates were developed in a stepwise fashion [multi-one-dimensional (MOD)-TLC] in chambers saturated with:

1. chloroform-methanol-water 65:40:5 (v/v/v) to 2 cm;
2. ethyl acetate-2-propanol-ethanol-chloroform-methanol-0.25% KCl 35:5:20:22:15:9 (v/v/v/v/v/v) to 5 cm;
3. toluene-diethyl ether-ethanol 60:40:3 (v/v/v) to 7.5 cm;
4. n-heptane-diethyl ether 94:8 (v/v) to 10.5 cm;
5. pure n-heptane to 12.5 cm.

A thorough drying of the plate between developments was extremely crucial. The disadvantage of their method is it is laborious. The main advantages of the present method over other conventional procedures were operational simplicity, rapidity, and sensitivity, with the maintenance of excellent reproducibility (Ruiz and Ochoa 1997). Obtained chromatogram according to Ruiz and Ochoa's procedure is shown in Figure 4.

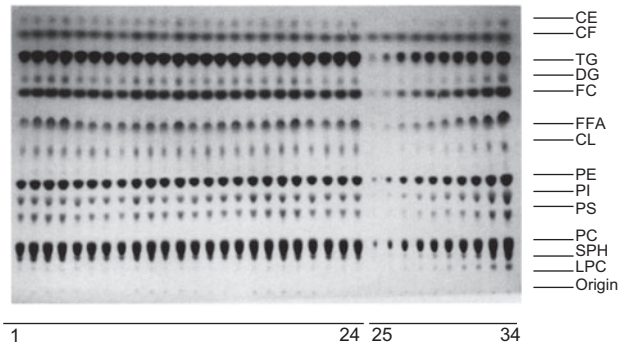

Figure 4 Representative thin-layer chromatogram of neutral lipids and PLs developed in stepwise fashion. A lipid extract portion (1 µl) from homogenates of eight different rat hepatocyte populations (lanes 1–8) was spotted in triplicate (lanes 9–16 and 17–24) on one EDTA-impregnated TLC plate. The lipid standards were spotted in lanes 25–34. Reprinted from Journal of Lipid Research, 38, Ruiz J. I., Ochoa B., "Quantification in the subnanomolar range of phospholipids and neutral lipids by monodimensional thin-layer chromatography and image analysis", 1482–1489, 1997 with permission from American Society for Biochemistry and Molecular Biology.

An analogous method for plate developing called MOD-TLC was used by White et al. for separation and quantification of PL species in mammalian cells and sera (White et al. 1998). Using the MOD-TLC system PC, PE, and PA were separated to homogeneity from lipid extracts of cultured ECV.304 cells and pooled human sera.

TLC could also be a useful method for separation of headgroup-modified PLs such as PE. PE was eluted with chloroform-methanol-acetic acid (80:12:8, v/v/v) and free fatty acids (FFAs) with diethyl ether-petrol ether-acetic acid (70:30:1, v/v/v). Air-dried plates were sprayed with ninhydrin and heated at 100°C to visualize the amine groups of PE or were charred at 180°C to visualize all lipids (Carr et al. 1998).

The separation of different PL classes can be improved by using two-dimensional (2D) TLC (Singh and Jiang 1995); a typical example from lipids of brain mitochondria and cream are shown in Figure 5 (MacKenzie et al. 2009).

2D TLC is much more efficient in separating PLs than 1D TLC, but this method has also disadvantages that significantly limit its applications. First, only a single sample can be investigated, and second, 2D TLC is much more time-consuming than 1D TLC. Furthermore, as only a single sample can be examined, a simultaneous application of standards is impossible. Thus, 2D TLC makes quantitative data analysis for PLs very difficult.

However, 2D TLC gives an excellent opportunity to separate a large number of PLs, as it was shown in a study by

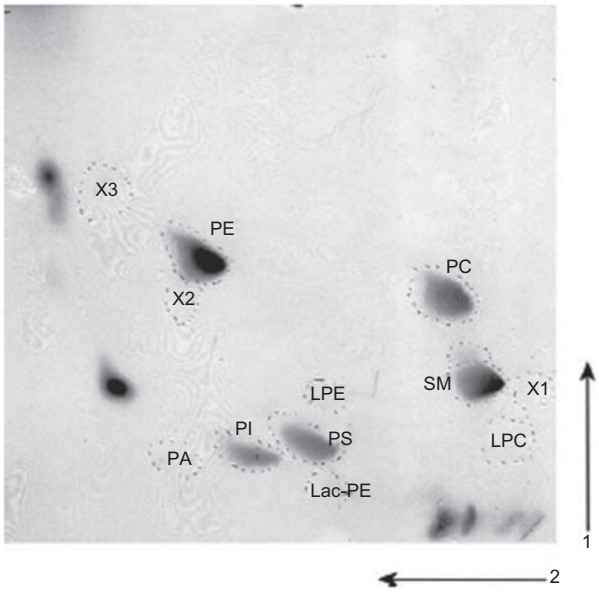

Figure 5 2D-TLC of cream polar lipids. Molybdate-positive spots were outlined and labeled. Solvent systems, 1st direction: chloroform-methanol-28% aq. NH_3-benzene (65:30:6:10, by vol.), 2nd direction: chloroform-ethyl acetate-acetone-2-propanol-absolute ethanol-methanol-water-acetic acid (30:6:6:6:16:28:6:2, by vol.). Reprinted from J. Am. Oil Chem. Soc., 86, MacKenzie et al., "Quantitative Analysis of Dairy Phospholipids by 31P NMR", 757–763, 2009, with permission from Springer (License number 2807650309292).

Eichenberger et al., where more than 100 different lipid species were separated and identified in brown algae (Eichenberger et al. 1993).

2D TLC is also an alternative if PLs are to be identified in a complex extract from cell membranes (Mallinger et al. 1993). Another successful use of extracts of *Clostridium butyricum* protoplasts could also be successfully separated by 2D TLC (Goldfine et al. 1993).

PL detection

Nondestructive methods

One of the first visualization methods for chromatograms was the use of iodine vapor, which forms brown noncovalent complexes with PLs (Nzai and Proctor 1998, Berczi and Horvath 2003). However, the above-mentioned method has two major disadvantages: first, PL spots are hardly visible in the presence of only saturated fatty acids in lipid molecules, and second, when unsaturated fatty acids are present in PLs, iodine may irreversibly bind to the double bonds (Vioque and Holman 1962, Hoving 1995). This technique of visualization is, nevertheless, one of the fastest and simplest nondestructive methods, making it extremely useful in assessing the quality of samples (Lattanzio et al. 2009).

Another method of visualization of a previously developed plate is based on spraying a solution containing 2,7-dichlorofluorescein or rhodamine 6G, which produces a yellow or pink spot, respectively, in UV light (Kuksis 2003). Rhodamine 6G is typically applied with alkaline mobile phases, while 2,7-dichlorofluorescein with acidic mobile phases; in both cases, the aim is to increase the stability of the dyes. Similar results are obtained by using primuline solution as a spraying reagent, where individual PLs became detectable as violet spots, and the detection limits obtained are of the order of nanomoles per spot (White et al. 1998, Sommerer et al. 2004, Richter et al. 2008).

The use of the above-mentioned reagents makes it possible to locate PLs on TLC plates and also to couple TLC with spectrophotometric (Skipski et al. 1964, Lattanzio et al. 2009) or matrix-assisted laser desorption/ionization time-of-flight mass spectrometric (MALDI-TOF MS) (Sommerer et al. 2004, Yurkova et al. 2004, Fuchs et al. 2007) methods for quantitative or qualitative analysis of PLs, respectively.

It has also been proven that polyunsaturated PLs show intense darkening when separation is performed on plates impregnated with $AgNO_3$, which may be explained as the consequence of the reduction of Ag^+ to colloidal silver (Martinez-Lorenzo et al. 1994). Interestingly, this darkening is dependent on the composition of the solvent system and seems to require the presence of aromatic hydrocarbons such as toluene.

Destructive nonspecific methods

PLs may also be visualized on TLC plates with destructive reagents. PL spots may be charred by spraying phosphomolybdic acid, sulfuric acid, or copper sulfate in phosphoric acid and then heating the plate. The resulting charred spots can be measured by densitometry (Skipski et al. 1963, Ruiz and Ochoa 1997, Klein et al. 1998, Grizard et al. 2000, Zhong et al. 2000, Weerheim et al. 2002). Another potentially useful method of visualization is spraying dried plates with a sulfuric acid-chromic acid spray and heating them in an oven at 180°C–200°C for 20 min (Cherayil and Scaria 1970).

Destructive specific methods

Currently, specific methods are applied increasingly often for PL determination. Here, the reagents react selectively with a specific PLs moiety to create a colored product. Table 1 presents the most frequently used reagents with a description of the results.

Examples of PL determination in biological samples

The use of reagents presented in Table 1 enables the determination of PLs in biological samples. Leray et al. have performed an efficient separation of all the main PL classes and quantified them in human blood by phosphorus assay or fatty acid analysis (Leray et al. 1987).

Another method for quantitating PLs separated on thin-layer chromatographic plates is by computer-assisted photodensitometry described by Vecchini et al. (1995). After development, the plates were stained with molybdic reagent, and the image obtained was acquired as TIFF file in the memory of a computer. The color intensity of the single spots of the digitalized images was analyzed using a highly specialized software. The dependence of the intensity of staining on the amount of PL was linear up to 1 μg of phosphorus, for each PL class. Sensitivity and reproducibility were adequate for most of the needs of a lipid chemist. The results reported by Vecchini et al. demonstrated that the developed quantitation procedure was more sensitive than the classical spectrophotometric method of Bartlett (1959), whereas, as far as sensitivity and reliability were concerned, the method of Baykov et al. (1988) and that described by Vecchini et al. appeared comparable. Another important advantage of the described method is that the scanned images, stored on a magnetic support, can be reprocessed many times, if needed.

The use of TLC immunostaining with the monoclonal antibody VJ-41, which preferentially reacts with SM and disaturated PC (DSPC), enables the detection of PLs in extracts from human amniotic fluid. This method is useful in the simultaneous selective determination of surfactant PLs and SM concentrations in the amniotic fluid to assess lung maturity in a fetus. More than 50 ng of dipalmityl PC and SM was detected on the same TLC plate, and the standard curves were linear up to 1 μg of PLs. The method was applied to determine the surfactant PC/SM ratio in 20 μl of amniotic fluid. The amniotic fluid of women who gave birth to children suffering from respiratory distress syndrome (RDS) was easily discriminated from the normal amniotic fluid (Iwamori et al. 1996).

Table 1 Visualization reagents most commonly used for the determination of PLs.

Reagent	Result/comments	Reference
Dragendorff reagent (bismuth nitrate+KI)	Orange-red spots of PC, LPC, SM	(Wagner et al. 1961)
Molybdic oxide/molybdenum	PLs formed blue spots on a white background, but the background was not so stable.	(Dittmer and Lester 1964)
α-Naphthol/sulfuric acid	All glycolipids formed yellow spots. Cholesterol was also reactive, but it provided a red spot.	(Kawanami 1967)
2′,7′-Dichlorofluorescein/$AlCl_3$/$FeCl_3$	Spots took a rose color after a few minutes.	(Dudziński 1967, Moe et al. 2004)
Ammonium molybdate/copper dissolved in concentrated hydrochloric acid	Blue spots on a white background, instability of the background.	(Vaskovsky and Kostetsky 1968)
Orcinol/sulfuric acid	PLs formed blue-purple spots on a white background.	(Neskovic et al. 1972)
Ammonium pentachlorooxomolybdate (APCOM) $(NH_4)_2[MoO_4]$	PLs showed up immediately, giving blue or sometimes greenish-blue spots that changed to blue completely after a few minutes, with the background remaining faintly yellow. The intensity of the blue spots increased on standing and became most intense after several hours. Fading started after 24 h, but the background color remained unchanged.	(Kundu et al. 1977)
Ammonium molybdate/molybdenum dissolved in hydrochloric acid/30% aqueous acetic acid	PLs gave sharp blue spots almost immediately. The intensity increased on standing in the atmosphere for approximately 10 min or after heating on a hot plate (60–70°C) for less than 10 s. An initial pale blue coloration of the background disappeared to give a uniform white background after approximately 3 min standing. Excessive application of the spray reagent could cause a colored (blue-green) background.	(Ryu and MacCoss 1979, Iorio et al. 2003)
1,6-Diphenyl-1,3,5-hexatriene (DPH)	Bluish-white fluorescent-stained PLs spots against a dark violet background under UV light (366 nm). The intensely fluorescent spots faded quickly and, thus, had to be photographed immediately after chromatography.	(Müthing and Radloff 1998)
Ammonium molybdate/perchloric acid	PLs turned blue gray on a white background after spraying with ammonium molybdate/perchloric acid spray reagent and heating the plate at 80°C for 10 min.	(Nzai and Proctor 1998, Sek et al. 2001)
10% Phosphomolybdic acid in 20% ethanol	Blue spots on a white background	(Zhong et al. 2000)
Ninhydrin in butanol	Red-violet spots from PE, PS, and the corresponding lysolipids.	(Valls et al. 2002)
3% Copper acetate	In order to visualize, the plate should be heated at 180°C by 10–15 min. The heating time should be adapted to the type of stationary phases.	(Grizard et al. 2000, Weerheim et al. 2002)
Thionine/Beibrich scarlet reagent	Acidic PLs formed violet spots (λ_{max}=600 nm), whereas alkenyl PL spots became green-blue (λ_{max}=625 nm), but also sulfatides formed violet spots (λ_{max}=600 nm).	(Helmy et al. 2002, Helmy 2004)

Ruiz and Ochoa presented a sensitive, fast, and simple method for PL quantification. For a simultaneous determination of the major PLs and neutral lipids, an *image analysis* technique was used. A clear-cut separation of the lipids was achieved on one EDTA-impregnated chromatoplate developed five times in a stepwise manner. The calibration curves for each lipid followed linear or hyperbolic functions. The method was suitable for routine analysis of biological samples with lipid profiles as different as rat hepatocyte subcellular fractions and very low density lipoproteins secreted in rat hepatocyte suspensions (Ruiz and Ochoa 1997). PLs were quantified in the range 0.015–4.43 nmol/spot. As only a tiny quantity of sample is needed to perform the analysis, this method is suitable for routine analysis of small amounts of biological materials, and it also allows the acyl profile of individual lipids to be studied by HPLC or GC after their resolution (Ruiz and Ochoa 1997).

As we mentioned earlier, White et al. used MOD-TLC for quantification of PC, PE, and PA in mammalian cells and sera (White et al. 1998). Mass spectral analysis done by researches revealed no contamination of one lipid class with another, supporting the quality of the separation that was obtained. For the rapid determination of lipid separation, the nondestructive lipophilic dye, primulin, and a handheld UV lamp to visualize the separated lipid bands were used. As White et al. proved, the fluorescent detection of primulin-dyed lipids was stable for several days, affording later analysis, if necessary.

As the detection method used by White et al. was nondestructive, purified lipids were then recovered by scraping the visualized bands and extracting the lipids from the silica (according to authors >95% of all lipid could be extracted from silica for further analysis). The structural identities of the recovered lipids were confirmed by fast-atom bombardment and electrospray MS. Extracted lipids were also hydrolyzed to release acyl chains, and acyl chain species were determined in comparison to authentic standards by GC. PA and diacylglycerol (DG) levels in cells were found to be 4.6% and 3.3%, respectively, of PC levels, with a PA/DG ratio of 1.4.

The presented method had many advantages:

a) A variety of solvent systems allowed for a clear separation of PLs with excellent separation of similar neutral glycerophospholipids, which represented a clear advantage over both conventional TLC and HPLC methods.
b) The analysis of lipid bands using primulin followed by fluoroimaging resulted in sensitive quantification at lower concentrations of PLs (in the range of nM lipid/mg ECV cell protein or nmol lipid/ml sera), thus reducing numbers of cells needed to analyze for changes in bioactive lipids.
c) Preservation of lipid mass by analytic technique such as primulin spray without charring allowed further analysis of lipid acyl composition by hydrolysis followed by GC analysis or by MS.

As TLC is a common method of lipid analysis, there are many reports where MALDI MS analysis was performed subsequent to TLC separation and scraping of the spots of interest. The lipid composition of body fluids (Sommerer et al. 2004), cells (Schiller et al. 2003a), and tissues (Schiller et al. 2003b) could be successfully analyzed by this approach.

An interesting PL determination was described by Sommerer et al. (2004). Samples of human and minipig lungs were used in this study, as they are known to differ in their PL composition. Surfactant PLs were separated by TLC, and the obtained subfractions were subjected to MALDI-TOF MS analysis in order to check for the presence of even minor PL species. It was shown that besides PG and PC, also PE, PI, and SM were detected in the human sample, whereas only SM was detected in the minipig sample.

Leßig et al. investigated the lipid composition of human and boar spermatozoa by TLC coupled with MALDI-TOF MS. Boar spermatozoa were characterized by a lower variability of their PLs and fatty acid composition (Leßig et al. 2004). These results were of considerable interest in the context of fertilization studies on human and boar spermatozoa, where high amounts of unsaturated fatty acids, such as docosahexaenoic and docosapentaenoic acids, were found to be essential in maintaining high membrane fluidity, which is required for successful fertilization (Zalata et al. 1998, Müller et al. 1999). One drawback is that PLs containing quaternary ammonia groups (in SM and PC) are detectable by positive ion MALDI-TOF MS, whereas PE is less sensitive to detection in mixtures containing comparable amounts of PC (Petković et al. 2001). Therefore, the prior separation of spermatozoa extracts by TLC represents a useful approach in detecting PLs in mixtures by MALDI-TOF MS.

Bavaro et al. also coupled a TLC technique with MS study on the lipid content of higher plants (spinach) under hyperosmotic stress. Quantitative analysis of PL content was performed by video densitometry using the software Image J (National Institute of Mental Health, Bethesda, MD, USA). The obtained results showed that hyperosmotic stress induced changes in PL content depending on the different plant organization levels studied. The results suggested an important role of PLs and, in particular of PG, in the osmotic stress response (Bavaro et al. 2007).

Fuchs et al. reported a method of directly coupling MALDI-TOF MS and TLC that can be easily implemented with commercially available MALDI-TOF devices and is shown in Figure 6. A total extract of hen egg yolk was used as a simple PL mixture to demonstrate the potential of this method, which was sensitive enough to detect the presence of PLs at levels of <1% of the total extract (Fuchs et al. 2007).

As we presented above, there have been some reports of TLC-MALDI regarding PLs. However, these studies were only performed with relatively simple PL mixtures, and there was no discussion of the reproducible and quantitative aspects of their method.

The first attempts to validate the quantitative and reproducible transfer of lipids from an HPTLC plate to the PVDF membrane with the use of MALDI-TOF MS were done by Goto-Inoue et al. (2009). Their results show that the limit of detection (LOD) of TLC-Blot-MALDI-TOF MS is as low as 5 pmol PC. Moreover, the calibration curve, which was made according to PC sodium-adduct $[M^+Na]^+$ ion intensities, showed sufficient linearity (R^2=0.9896). This method was

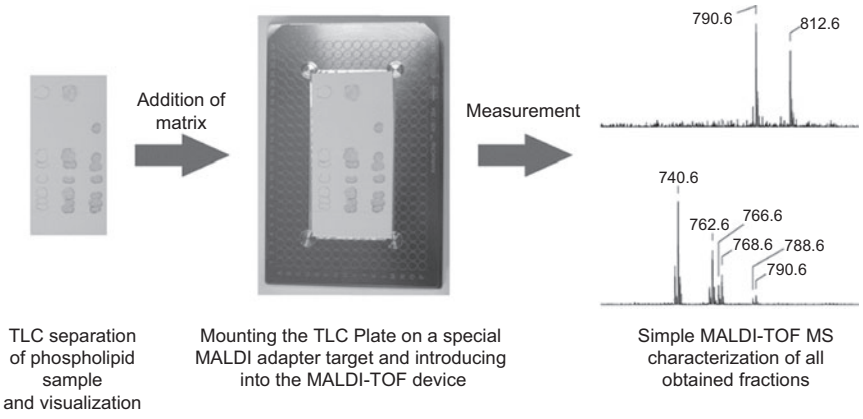

Figure 6 The general scheme for coupling TLC with MALDI. TLC/MALDI coupling was performed in the following way: PLs were separated by TLC in the conventional way and stained with the dye primuline that bound noncovalently to the acyl residues of PLs. Then, the spots of interest were soaked with matrix and the TLC plate mounted onto a special adapter target. This target was introduced into the mass spectrometer and analyzed in the conventional manner. Reprinted from Anal. Bioanal. Chem., 389, Fuchs B., et al., "A direct and simple method of coupling matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS) to thin-layer chromatography (TLC) for the analysis of phospholipids from egg yolk", 827–834, 2007 with permission from Springer (License number 2810810331085).

applied to total lipids extracted from human brain samples (see Figure 7).

What is more, the researchers were the first who used this method to the PL samples from four different sites, white and gray matter of both the inferior frontal gyrus and the hippocampus. Using this new method, they demonstrated the differential display of PLs among the different positions of the brain and identified lipids having a specific distribution. The results showed that the amounts of SM differed significantly between the gray and white matter. In the optical image, they saw that all SM molecules were abundant in the white matter compared with the gray matter. They were able to identify a region-specific distribution of individual lipids in terms of their molecular species by the use of TLC-Blot-MALDI MS.

As we presented, the use of MALDI-TOF MS increased the sensitivity and resolution of TLC analysis of PLs. A special MALDI adapter target, used by Fuchs et al. (2007) made this method competitive with much more sophisticated, much more expensive, and more time-consuming methods of PL analysis.

Fatty acid determination

Another important aspect in PL determination is detection and determination of fatty acids, which are also presented next to PLs in biological samples. Fatty acids can be found in huge amounts in fat tissues of the organism although most of them are esterified with alcohols, i.e., glycerol. Apart from their fundamental role as a fuel for energy production, fatty acids are incorporated into PLs forming the core of biological membranes and serve in selected signal transduction pathways to alter gene expression. However, largely due to their hydrophobic properties, fatty acids also exert harmful effects and may cause (acute) cellular injury (Wang et al. 2009). That is why amount of FFAs next to PLs can be important markers of many diseases, like atherosclerosis, heart or cardiovascular diseases (Leitinger 2008).

Free polyunsaturated fatty acids could be easily separated from lipids by normal phase TLC where Silica gel 60 TLC plates coated with $AgNO_3$ were used. The plates were developed with mobile phase made by mixing toluene and hexane in a volume ratio of 40:60. Separated fatty acid methyl esters (FAMEs) were visualized by charring, autoradiography, or under UV light after spraying with dichlorofluorescein (Wilson and Sargent 2001). This method allowed to separate monounsaturated FAMEs according to chain length. This technique could be applied to study the elongation of [1-^{14}C]-oleic acid or desaturation of [1-^{14}C]-stearic acid in cells in culture. According to the authors, the argentation TLC technique is simple and inexpensive in comparison to radio-GC and it could also be an excellent alternative and complementary method to GC and HPLC in studying elongation of monounsaturated and/or desaturation of saturated fatty acids.

Schwertner and Mosser (1993) showed not only qualitative but also quantitative determination of different fatty acids using butylated hydroxytoluene (BHT) as antioxidant. The total lipid extract was separated on silica gel with n-hexane-diethyl ether-acetic acid-BHT (95:5:1:0.1, v/v/v/v); then, chromatoplates were sprayed with rhodamine 6G and the individual lipid classes monitored under UV light. GC was used for quantitation. According to the authors, serum concentrations of lipid fatty acids were more accurate reflection of changes in lipid fatty acids than were weight percentage calculations and were more easily interpreted in metabolic and therapeutic terms.

As was described above, nowadays, more often for lipid and fatty acid analysis, TLC coupled with other techniques, like GC or flame ionization detector (FID), is used.

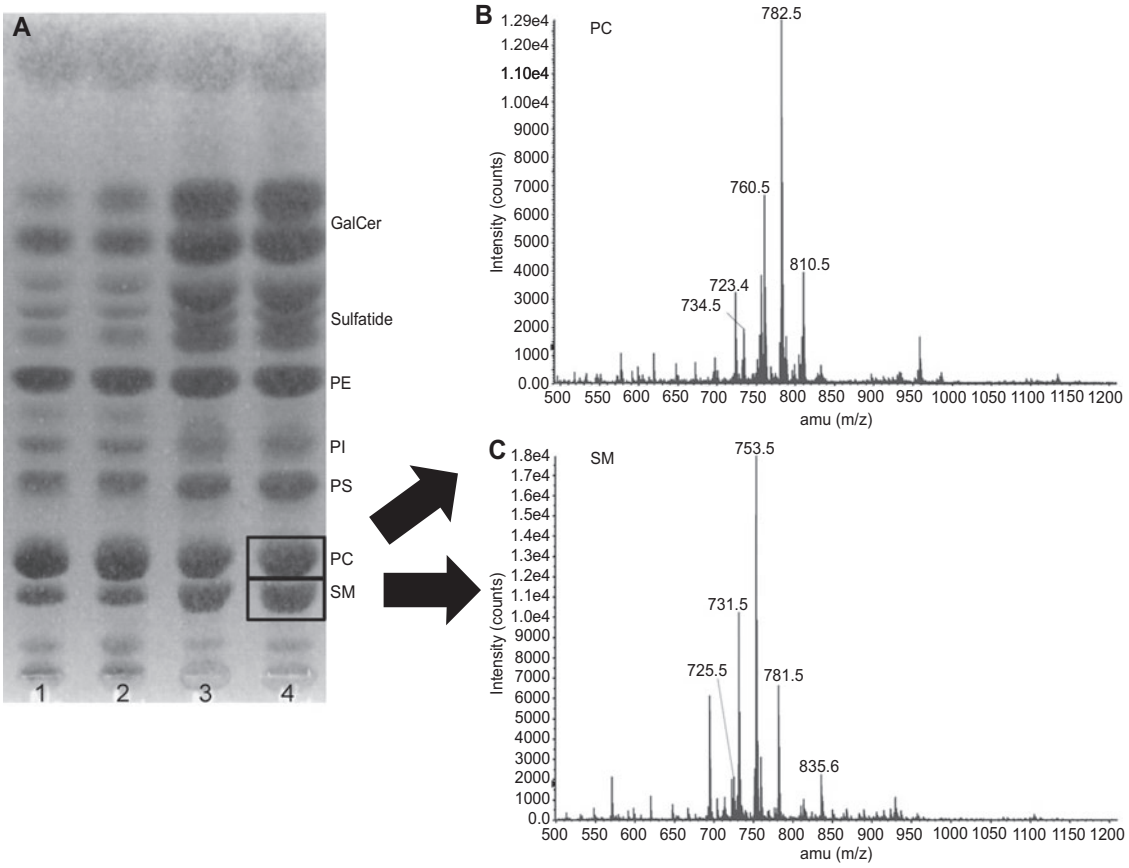

Figure 7 A typical chromatogram for quantitative determination of major PLs with the use of TLC coupled with imaging mass spectrometry: (A) Thin-layer chromatogram stained with primuline. Lanes (1–4) contain human brain lipid extracted from gray matter of inferior frontal gyrus (lane 1), gray matter of hippocampus (lane 2), white matter of inferior frontal gyrus (lane 3), and white matter of hippocampus (lane 4). The MS spectra of PC (B) and SM (C) allowed to directly analyze the blotted PVDF membrane. Reprinted from Journal of Chromatography A, 1216, Goto-Inoue N., Hayasaka T., Taki T., Valdes Gonzalez T., Setou M., "A new lipidomics approach by thin-layer chromatography-blot-matrix-assisted laser desorption/ionization imaging mass spectrometry for analyzing detailed patterns of phospholipid molecular species" 7096–7101, 2009, with permission from Elsevier (License number 2807110042553).

Rosas-Romero and coworkers contributed to a new procedure for quantitative analysis of lipid classes of interest to the fat and oil industries (Rosas-Romero et al. 1994, 1996). But the results were not rewarding because of the lack of linearity; that is why the researchers suggested that there is a need to establish an arithmetic transformation of data. In order to improve quantitation of lipid classes, they created a correlation between the log of the peak-area ratios against the log of weight ratio (Rosas-Romero et al. 1996).

Also, a simple method for lipid determination in cooked beef with the use of TLC-FID was developed by St Angelo and James (1993). This simple procedure consisted of a first development with the solvent system benzene-chloroform-formic acid (50:20:1.5, v/v/v) followed by a short development with a mixture of chloroform-methanol-29% ammonium hydroxide (50:50:5, v/v/v), then the full scan. The first development separated triglycerides (TGs) and cholesterol, whereas the second provided good resolution of all major PLs and lysophospholipids.

Cavalier et al. created a rapid and reliable determination method for each class of lipids by TLC-FID (Cavalier et al. 2009). Of each organic lipid extract, 1.0 µl (obtained according to the Folch's method) was spotted onto silica-coated quartz rods (SIII Chromarods), and the elution was performed with n-heptane-diethyl ether-formic acid (55:45:1, v/v/v) – a solvent mixture that allowed, according to Carrière et al. (2001), the separation of all TG lipolysis products in a single step. The linearity of the presented method was confirmed over the whole working concentration range of 0.400–10.0 mg/ml for all class of lipids – TG, FFA, DG, and monoacylglycerol (MG). This method allowed quantitative determination of each class of lipids in human gastrointestinal fluid. It should also be noticed that this method required only a single elution scheme to analyze TG and their lipolysis products (DG, MG, FFA).

There is also a report that presented fatty acid determination by TLC in biodiesel. Hot acidic p-anisaldehyde was used to specifically stain lipid contaminants such as TG, DG, and

MG in biodiesel, and good agreement with simultaneously obtained GC data was achieved. However, detection limits achievable by this approach were rather poor because these acylglycerols were detectable by the proposed planar chromatographic method, provided the content of the contaminants exceeds the limits recommended by the international norms applicable to biodiesel quality/specification, namely, 0.25% in mass for total combined glycerin. The presented technique had also advantages, namely, it was fast and low cost for monitoring main lipid contaminants in biodiesel (Fontana et al. 2009).

Mougios and Petridou presented methods for the analysis of the main lipid classes in skeletal muscles of humans and other animals. In these methods, lipids were extracted from muscle according to Folch's procedure, and they were separated by TLC; then, lipids were subsequently subjected to methanolysis according to Kramer et al. (1997) to produce methyl esters of fatty acids (and, to a lesser extent, dimethyl acetals of fatty aldehydes derived from plasmalogens), which were analyzed by GC.

Also, 2D TLC could be successfully used for the separation of FFAs. As Rezanka showed, using one part of layer impregnated with urea (first dimension of development) and the second part with $AgNO_3$ (second dimension) allowed the separation of FAMEs according to the structure of the chain (branched) and also by the number of their double bonds (Rezanka 1996). The above-mentioned method was employed to separate a natural FAME mixture. The mixture was obtained from cod liver oil, known for the complex nature of the fatty acids present. In this case, to reach an even higher complexity of the analyzed mixture, the author also used FAMEs from *Streptomyces avermitilis* bacteria because they are well known for their production of *iso* acids and *anteiso* acids. The plate was developed with butyl acetate in the first direction, then turned around by 90° and developed with a mixture of hexane, diethyl ether, and methanol (90:10:1, v/v/v), where the polar solvent had to be present to disrupt the urea complex. After desiccation, the plate was sprayed with a 0.1% ethanol solution of 2′,7′-dichlorofluorescein and the corresponding spots marked under UV light. The silica gel was scraped and extracted with a mixture of hexane-diethyl ether (1:1, v/v), and after evaporation, the FAMEs were converted into oxazolines according to Yu et al. (1988). For identification of oxazolines, GC-MS was used. The main advantage of the above-mentioned method is the possibility of semipreparation of the individual tractions of fatty acids, even when the initial amount of the compound is in the range of only micrograms. This situation occurs frequently, especially when FAMEs from not very common animals or plants are being analyzed.

Summary

The aim of this review was to illustrate potential applications of TLC regarding PL analysis and also to discuss some useful methods of PL extraction and TLC separation. TLC could be

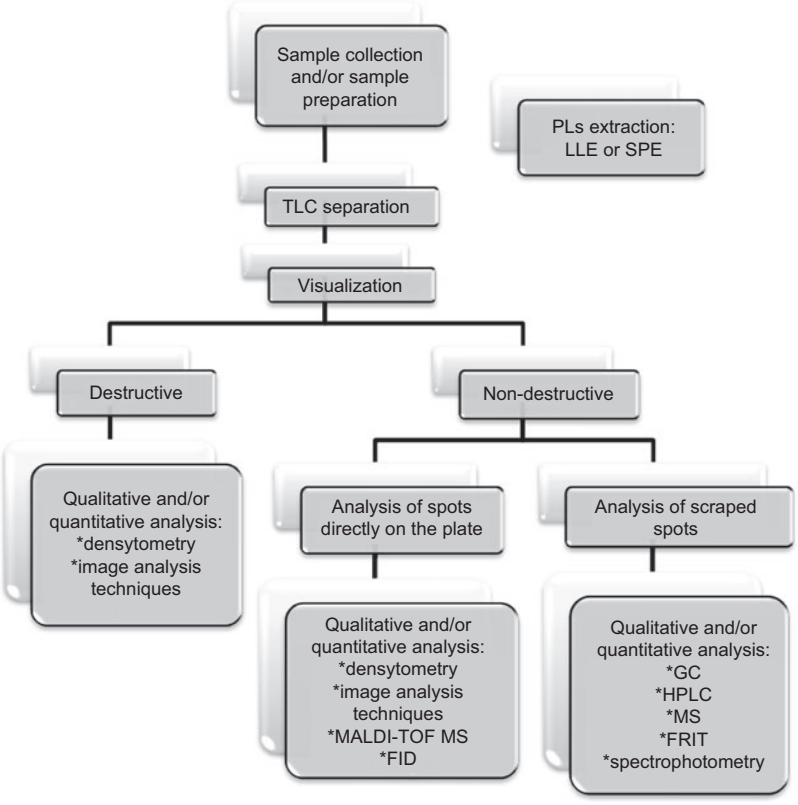

Figure 8 The general scheme for PL determination with the use of TLC.

used most often when a single sample whose purity or identity needs to be checked. It is often tedious, time-consuming, and costly in terms of solvents to do this by HPLC or GC.

The selection of the most appropriate method for PL determination depends mainly on the analytical problem, which we want to solve, and the available equipment. Figure 8 presents the general scheme for PL determination with the use of TLC.

If we want to do qualitative or simple and fast quantitative analysis of the samples containing PL, TLC with the destructive visualization systems may be an excellent choice. But when we need to know more about tested samples, TLC with nondestructive detection system, also coupled with other techniques, like GC, MS, or HPLC, could be more suitable. The use of nonspecific visualization reagents, such as primuline spray, also allows the acyl profile of individual lipids to be studied.

TLC allows for quick and inexpensive separation of different PL classes. The spray reagents presented in this paper enable quantitative PL determination and, therefore, constitute an alternative to more expensive techniques, such as HPLC, GC, or MS.

The use of TLC in PL determination offers:

- Low cost and uses small quantities of solvents;
- Parallel separation of many samples with minimal time requirement;
- Unsurpassed clarity and simultaneous visual evaluation of all samples and sample components;
- Simplified sample preparation due to single use of the stationary phase;
- Possibility of multiple evaluations of the plate with different parameters because all fractions of the sample are stored on the plate.

In our opinion, in skilled hands, TLC offers considerable versatility and precision in lipid analysis with relatively low capital costs; that is why, nowadays, results obtained using TLC can compete with other instrumental techniques.

However, the use of TLC for PL analysis has also several disadvantages. First, the use of the most common spray reagents can destroy the PL structure (Pulfer and Murphy 2003), but this can also occur when using alternative methods (Horning et al. 1971, Devle et al. 2011). Second, although TLC allows for the quick separation of PLs, this method is not automated like GC or HPLC, which is why the analyst must perform particular steps manually.

The results show that a qualitative analysis of PL classes can be performed by TLC technique, even if the samples contain also other polar and nonpolar components.

Given the technological advances and the falling prices of equipment and reagents, it seems that TLC may become a tool used in clinical laboratories for diagnosing many diseases involving changes in PL signaling.

References

Abidi, S. L. Separation procedures for phosphatidylserine. *J. Chromatogr. B Biomed. Sci. Appl.* **1998**, *717*, 279–293.

Alberts, B.; Bray, D.; Hopkin, K.; Johnson, A.; Lewis, J.; Raff, M.; Roberts, K.; Walter, P. Essential Cell Biology; 2nd Edition. Garland Science/Taylor & Francis Group: London, UK, 2004.

Allan, D.; Cockcroft, S. A modified procedure for thin-layer chromatography of phospholipids. *J. Lipid Res.* **1982**, *23*, 1373–1374.

Ando, S.; Saito, M. Chromatography of lipids. In: Biomedical Research and Clinical Diagnosis. Kuksis, A., Ed. Elsevier: Amsterdam, 1987, pp. 266–310.

Asadi, F.; Rostami, A.; Pourkabir, M.; Shahriari, A. Serum lipid and lipoprotein profile of Asian tortoise (*Agrionemys horsfieldi*) in prehibernation state. *Comp. Clin. Path.* **2007**, *16*, 193–195.

Avalli, A.; Contarini, G. Determination of phospholipids in dairy products by SPE/HPLC/ELSD. *J. Chromatogr. A* **2005**, *1071*, 185–190.

Banni, S.; Carta, G.; Angioni, E.; Muru, E.; Scanu, P.; Melis, M. P.; Bauman, D. E.; Fischer, S. M.; Ip, C. Distribution of conjugated linoleic acid and metabolites in different lipid fraction in the rat liver. *J. Lipid Res*. **2001**, *42*, 1056–1061.

Bartlett, G. R. Phosphorus assay in column chromatography. *J. Biol. Chem.* **1959**, *234*, 466–468.

Bavaro, L.; Catucci, L.; Depalo, N.; Ventrella, A.; Corcelli, A.; Agostiano, A. Lipid content in higher plants under osmotic stress. *Bioelectrochemistry* **2007**, *70*, 12–17.

Bayir, H.; Tyurin, V. A.; Tyurina, Y. Y.; Viner, R.; Ritov, V.; Amoscato, A. A.; Zhao, Q.; Zhang, X. J.; Janesko-Feldman, K. L.; Alexander, H.; Basova, L. V.; Clark, R. S.; Kochanek, P. M.; Kagan, V. E. Selective early cardiolipin peroxidation after traumatic brain injury: an oxidative lipidomics analysis. *Ann. Neurol.* **2007**, *62*, 154–169.

Baykov, A. A.; Evtushenko, O. A.; Aveava, S. M. A malachite green procedure for orthophosphate determination and its use in alkaline phosphate-based enzyme immunoassay. *Anal. Biochem.* **1988**, *171*, 266–271.

Berczi, A.; Horvath, G. Lipid rafts in the plant plasma membrane? *Acta Biol. Szeged.* **2003**, *47*, 7–10.

Bevers, E. M.; Comfurius, P.; Dekkers, D. W.; Harmsma, M.; Zwaal, R. F. Transmembrane phospholipid distribution in blood cells: control mechanisms and pathophysiological significance. *Biol. Chem.* **1998**, *379*, 973–986.

Bligh, E. G.; Dyer, W. J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **1959**, *37*, 911–917.

Bodera, P. Wspomaganie pamięci – nowe trendy, Świat farmacji – Farmakoterapia. *ang.: Memory Power – new trends, World Pharmacy – Pharmacotherapy,* Pub.: *Apteka* **2009**, *1*, 14–17.

Carr, A. C.; van den Berg, J. J.; Winterbourn, C. C. Differential reactivities of hypochlorous and hypobromous acids with purified *Escherichia coli* phospholipid: formation of haloamines and halohydrins. *Biochim. Biophys. Acta* **1998**, *1392*, 254–264.

Carrière, F.; Renou, C.; Ransac, S.; Lopez, V.; De Caro, J.; Ferrato, F.; De Caro, A.; Fleury, A.; Sanwald-Ducray, P.; Lengsfeld, H.; Beglinger, C.; Hadvary, P.; Verger, R.; Laugier, R. Inhibition of gastrointestinal lipolysis by Orlistat during digestion of test meals in healthy volunteers. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2001**, *281*, G16–G28.

Cavalier, J.-F.; Lafont, D.; Boullanger, P.; Houisse, D.; Giallo, J.; Ballester, J.-M.; Carrière, F. Validation of lipolysis product extraction from aqueous/biological samples, separation and quantification by thin-layer chromatography with flame ionization detection analysis using *O*-cholesteryl ethylene glycol as a new internal standard. *J. Chromatogr. A* **2009**, *1216*, 6543–6548.

Cherayil, G. D.; Scaria, K. S. Thin-layer chromatography of tissue lipids without extraction. *J. Lipid Res.* **1970**, *11*, 378–381.

Christie, W. W. Structural analysis of lipids by means of enzymatic hydrolysis, In: Lipid Analysis. Christie, W. W., Ed. Pergamon Press: New York, 1982, pp. 155–166.

De Schrijver, R.; Vermeulen, D. Separation and quantitation of phospholipids in animal tissues by latroscan TLC/FID. *Lipids* **1991**, *26*, 74–76.

Devle, H.; Naess-Andresen, C. F.; Stenstrøm, Y.; Ekeberg, D. Rapid method for analysis of sphingomyelin by microwave derivatisation for gas chromatography-mass spectrometry. *Eur. J. Lipid Sci. Technol.* **2011**, *113*, 708–710.

Dittmer, J. C.; Lester, R. L. A simple specific spray for the detection of phospholipids on thin-layer chromatograms. *J. Lipid Res.* **1964**, *5*, 126–127.

Donato, P.; Cacciola, F.; Cichello, F.; Russo, M.; Dugo, P.; Mondello, L. Determination of phospholipids in milk samples by means of hydrophilic interaction liquid chromatography coupled to evaporative light scattering and mass spectrometry detection. *J. Chromatogr. A* **2011**, *1218*, 6476–6482.

Dudziński, A. E. A spray sequence specific for the detection of free fatty acids. *J.* Chromatogr. **1967**, *31*, 560–561.

Eichenberger, W.; Araki, S.; Muller, D. G. Betaine lipids and phospholipids in brown alga. *Phytochemistry* **1993**, *34*; 1323–1333.

Flurkey, W. H. Use of solid phase extraction in the biochemistry laboratory to separate different lipids. *Biochem. Mol. Biol. Educ.* **2005**, *33*, 357–360.

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

Fontana, J. D.; Zagonel, G.; Vechiatto, W. W.; Costa, B. J.; Laurindo, J. C.; Fontana, R.; Pelisson, L.; Jorge, B. H.; Lancas, F. M. Simple TLC-screening of acylglycerol levels in biodiesel as an alternative to GC determination. *J. Chromatogr. Sci.* **2009**, *47*, 844–846.

Fuchs, B.; Schiller, J.; Süß, R.; Schürenberg, M.; Suckau, D. A direct and simple method of coupling matrix-assisted laser desorption and ionization time-of-flight mass spectrometry MALDI-TOF MS to thin-layer chromatography TLC for the analysis of phospholipids from egg yolk. *Anal. Bioanal. Chem.* **2007**, *389*, 827–834.

Goldfine, H.; Johnston, N. C.; Knob, C. Nonspecific phospholipase C of *Listeria monocytogenes*: activity on phospholipids in Triton X-100-mixed micelles and in biological membranes. *J. Bacteriol.* **1993**, *175*, 4298–4306.

Gorgas, K.; Teigler, A.; Komljenovic, D.; Just, W. W. The ether lipid-deficient mouse: tracking down plasmalogen functions. *Biochim. Biophys. Acta* **2006**, *1763*, 1511–1526.

Goto-Inoue, N.; Hayasaka, T.; Taki, T.; Valdes Gonzalez, T.; Setou, M. A new lipidomics approach by thin-layer chromatography-blot-matrix-assisted laser desorption/ionization imaging mass spectrometry for analyzing detailed patterns of phospholipid molecular species. *J. Chromatogr. A* **2009**, *1216*, 7096–7101.

Grizard, G.; Sion, B.; Bauchart, D.; Boucher, D. Separation and quantification of cholesterol and major phospholipid classes in human semen by high-performance liquid chromatography and light-scattering detection. *J. Chromatogr. B Biomed. Sci. Appl.* **2000**, *740*, 101–107.

Han, X.; Gross, R. W. Shotgun lipidomics: electrospray ionization mass spectrometric analysis and quantitation of cellular lipidomes directly from crude extracts of biological samples. *Mass Spectrom. Rev.* **2004**, *24*, 367–412.

Helmy, F. M. Comparative studies of the endogenous phospholipids and their *in vitro* hydrolysis by endogenous phospholipases of various tissues from 7-day-old chicks: a thin layer chromatographic and densitometric analysis. *Cell Biochem. Funct.* **2004**, *22*, 389–398.

Helmy, F. M.; Mueller, T. E.; Juracka, A. Determination of the phospholipid profile of *E. coli* by TLC with thionine as spot-test reagent. Recognition of cardiolipin, phosphatidylglycerol, phosphatidylethanolamine, and *N*-acyl phosphatidylethanolamine. *J. Planar Chromatogr.-Mod. TLC* **2002**, *15*, 23–27.

Honeyman, T. W.; Strohsnitter, W.; Scheid, C. R.; Schimmel, R. J. Phosphatidic acid and phosphatidylinositol labelling in adipose tissue. The role of endogenously formed adenosine. *Biochem. J.* **1983**, *212*, 489–506.

Horning, M. G.; Murakami, S.; Horning, E. C. Analyses of phospholipids, ceramides, and cerebrosies by gas chromatography and gas chromatography–mass spectrometry. *Am. J. Clin. Nutr.* **1971**, *24*, 1086–1096.

Hoving, E. B. Chromatographic methods in the analysis of cholesterol and related lipids. *J. Chromatogr. B Biomed. Appl.* **1995**, *671*, 341–362.

Iorio, E.; Di Vito, M.; Spadaro, F.; Ramoni, C.; Lococo, E.; Carnevale, R.; Lenti, L.; Strom, R.; Podo F. Triacsin C inhibits the formation of 1H NMR-visible mobile lipids and lipid bodies in HuT 78 apoptotic cells. *Biochim. Biophys. Acta* **2003**, *1634*, 1–14.

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

Iwamori, M.; Hirota, K.; Utsuki, T.; Momoeda, K.; Ono, K.; Tsuchida, Y.; Okumura, K.; Hanaoka, K. Sensitive method for the determination of pulmonary surfactant phospholipid/sphingomyelin ratio in human amniotic fluids for the diagnosis of respiratory distress syndrome by thin-layer chromatography-immunostaining. *Anal. Biochem.* **1996**, *238*, 29–33.

Kaulen, H. D. Separation of phosphatidylserine and -inositol by one-dimensional thin-layer chromatography of lipid extracts. *Anal. Biochem.* **1972**, *45*, 664–667.

Kawanami, J. Lipid of cancer tissues: II. Neutral glycolipids of Nakahara-Fukuoka sarcoma tissue. *J. Biochem.* **1967**, *62*, 105–117.

Kim, H.-Y.; Salem, N. Jr. Separation of lipid classes by solid phase extraction. *J. Lipid Res.* **1990**, *31*, 2285–2289.

Klein, T. R.; Kirsch, D.; Kaufmann, R.; Riesner, D. Prion rods contain small amounts of two host sphingolipids as revealed by thin-layer chromatography and mass spectrometry. *J. Biol. Chem.* **1998**, *379*, 655–666.

Kosicek, M.; Kirsch, S.; Bene, R.; Trkanjec, Z.; Titlic, M.; Bindila, L.; Peter-Katalinic, J.; Hecimovic, S. Nano-HPLC-MS analysis of phospholipids in cerebrospinal fluid of Alzheimer's disease patients – a pilot study. *Anal. Bioanal. Chem.* **2010**, *398*, 2929–2937.

Kramer, J. K. G.; Fellner, V.; Dugan, M. E. R.; Sauer, F. D.; Mossoba, M. M.; Yurawecz, M. P. Evaluating acid and base catalysts in the methylation of milk and rumen fatty acids with special emphasis on conjugated dienes and total trans fatty acids. *Lipids* **1997**, *32*, 1219–1228.

Kuksis, A. Inositol phospholipid metabolism and phosphatidyl inositol kinases. In: Laboratory Techniques in Biochemistry and Molecular Biology. Elsevier: Amsterdam, 2003, 42–47.

Kundu, S. K.; Chakravarty, S.; Bhaduri, N.; Saha, H. K. A novel spray reagent for phospholipid detection. *J. Lipid Res.* **1977**, *18*, 128–130.

Lattanzio, V. M. T.; Baronio, M.; Oren, A.; Russell, N. J.; Corcelli, A. Characterization of polar membrane lipids of the extremely halophilic bacterium *Salinibacter ruber* and possible role of cardiolipin. *Biochim. Biophys. Acta* **2009**, *1791*, 25–31.

Leitinger, N. The role of phospholipid oxidation products in inflammatory and autoimmune diseases: evidence from animal models and in humans. *Subcell. Biochem.* **2008**, *49*, 325–350.

Leray, C.; Pelletier, X.; Hemmendinger, S.; Cazenave, J-P. Thin-layer chromatography of human platelet phospholipids with fatty acid analysis. *J. Chromatogr.* **1987**, *420*, 411–416.

Leßig, J.; Fuchs, B. Plasmalogens in biological systems: their role in oxidative processes in biological membranes, their contribution to pathological processes and aging and plasmalogen analysis. *Curr. Med. Chem.* **2009**, *16*, 2021–2041.

Leßig, J; Gey, C.; Süß, R.; Schiller, J.; Glander, H.-J.; Arnhold, J. Analysis of the lipid composition of human and boar spermatozoa by MALDI-TOF mass spectrometry, thin layer chromatography and ^{31}P NMR spectroscopy. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **2004**, *137*, 265–277.

MacKenzie, A.; Vyssotski, M.; Nekrasov, E. Quantitative analysis of dairy phospholipids by ^{31}P NMR. *J. Am. Oil Chem. Soc.* **2009**, *86*, 757–763.

Mallinger, A. G.; Yao, J. K.; Brown, A. S.; Dippold, C. S. Analysis of complex mixtures of phospholipid classes from cell membranes using two-dimensional thin-layer chromatography and scanning laser densitometry. *J. Chromatogr. Biomed. Appl.* **1993**, *614*, 67–75.

Martinez-Lorenzo, M. J.; Marzo, I.; Naval, J.; Piñeiro, A. Self-staining of polyunsaturated fatty acids in argentation thin-layer chromatography. *Anal. Biochem.* **1994**, *220*, 210–212.

Moe, M. K.; Anderssen, T.; Strom, M. B.; Jensen, E. Vicinal hydroxylation of unsaturated fatty acids for structural characterization of intact neutral phospholipids by negative electrospray ionization tandem quadrupole mass spectrometry. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 2121–2130.

Moser, A. B.; Steinberg, S. J.; Watkins, P. A.; Moser, H. W.; Ramaswamy, K.; Siegmund, K. D.; Lee, D. R.; Ely, J. J.; Ryder, O. A.; Hacia, J. G. Human and great ape red blood cells differ in plasmalogen levels and composition. *Lipids Health Dis.* **2011**, *10*, 101–114.

Müller, K.; Pomorski, T.; Müller, P.; Herrmann, A. Stability of transbilayer phospholipid asymmetry in viable ram sperm cells after cryotreatment. *J. Cell Sci.* **1999**, *112*, 11–20.

Müthing, J.; Radloff, M. Nanogram detection of phospholipids on thin-layer chromatograms. *Anal. Biochem.* **1998**, *257*, 67–70.

Myher, J. J.; Kuksis, A. General strategies in chromatographic analysis of lipids. *J. Chromatogr. B Biom. Appl.* **1995**, *671*, 3–33.

Nagan, N.; Zoeller, R. A. Plasmalogens: biosynthesis and functions. *Prog. Lipid Res.* **2001**, *40*, 199–229.

Neskovic, N.; Sarlieve, L.; Nussbaum, J.-L.; Kostic, D.; Mandel, P. Quantitative thin-layer chromatography of glycolipids in animal tissues. *Clin. Chim. Acta* **1972**, *38*, 147–153.

Nzai, J. M.; Proctor, A. Phospholipids determination in vegetable oil by thin-layer chromatography and imaging densitometry. *Food Chem.* **1998**, *63*, 571–576.

Perez-Palacios, T.; Ruiz, J.; Antequera, T. Improvement of a solid phase extraction method for separation of animal muscle phospholipid classes. *Food Chem.* **2007**, *102*, 875–879.

Petković, M.; Schiller, J.; Müller, M.; Benard, S.; Reichl, S.; Arnold, K.; Arnhold, J. Detection of individual phospholipids in lipid mixtures by matrix assisted laser desorption/ionization time-of-flight mass spectrometry: phosphatidylcholine prevents the detection of further species. *Anal. Biochem.* **2001**, *289*, 202–216.

Pietsch, A.; Reinhard, L. L. Rapid separation of the major phospholipid classes on a single aminopropyl cartridge. *Lipids* **1993**, *28*, 945–947.

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

Radin, N. S. Extraction of tissue lipids with a solvent of low toxicity. *Methods Enzymol.* **1981**, *72*, 5–7.

Radziwon, P.; Kłoczko, J.; Kiss, B. Współczesna teoria aktywacji i kontroli krzepni cia krwi. *ang.: Modern activation and coagulation control theory of blood*, Ed: *Przew. Lek.* **2004**, *11*, 50–56.

Rastogi, S. C. Lipids in Cell Biology, 3rd Edition. New Age International: New Delhi, India, 2005, pp. 50–54.

Rezanka, T. Two-dimensional separation of fatty acids by thin-layer chromatography on urea and silver nitrate silica gel plates. *J. Chromatogr. A* **1996**, *727*, 147–152.

Richter, G.; Schober, C.; Süß, R.; Fuchs, B.; Müller, M.; Schiller, J. The reaction between phosphatidylethanolamines and HOCl investigated by TLC: Fading of the dye primuline is induced by dichloramines. *J. Chromatogr. B* **2008**, *867*, 233–237.

Rosas-Romero, A. J.; Herrera, J. C.; Martinez de Aparicio, E. Thin-layer chromatographic determination of β-carotene, cantaxanthin, lutein, violaxantin and neoxantin on Chromarods. *J. Chromatogr. A* **1994**, *667*, 361–266.

Rosas-Romero, A. J.; Herrera, J. C.; Muccini, M. Determination of neutral and polar lipids by thin-layer chromatography with flame ionization detector TLC-FID. *Ital. J. Food Sci.* **1996**, *1*, 33–39.

Ruiz, J. I.; Ochoa, B. Quantification in the subnanomolar range of phospholipids and neutral lipids by one-dimensional thin-layer chromatography and image analysis. *J. Lipid Res.* **1997**, *38*, 1482–1489.

Ruiz-Gutierrez, V.; Perez-Camino, M. C. Update on solid-phase extraction for the analysis of lipid classes and related compounds. *J. Chromatogr. A* **2000**, *885*, 321–341.

Ryu, E. K.; MacCoss, M. Modification of the Dittmer-Lester reagent for the detection of phospholipid derivatives on thin-layer chromatograms. *J. Lipid Res.* **1979**, *20*, 561–563.

Sapa, A. The usefulness of contemporary laboratory medicine in fetal lung maturity assessment. *Adv. Clin. Exp. Med.* **2006**, *15*, 345–353.

Schiller, J.; Müller, K.; Suss, R.; Arnhold, J.; Gey, C.; Herrmann, A.; Lessig, J.; Arnold, K.; Muller, P. Analysis of the lipid composition of bull spermatozoa by MALDI-TOF mass spectrometry – a cautionary note. *Chem. Phys. Lipids* **2003a**, *126*, 85–94.

Schiller, J.; Süß, R.; Fuchs, B.; Müller, M.; Zschörnig, O.; Arnold, K. Combined application of TLC and matrix-assisted laser desorption and ionisation time-of-flight mass spectrometry MALDI-TOF MS to phospholipid analysis of brain. *Chromatographia* **2003b**, *57*, S297–S302.

Schiller, J.; Suss, R.; Arnhold, J.; Fuchs, B.; Leszig, J.; Muller, M.; Petkovic, M.; Spalteholz, H.; Zschornig, O.; Arnold, K. Matrix assisted laser desorption and ionization time-of-flight (MALDI-TOF) mass spectrometry in lipid and phospholipid research. *Prog. Lipid Res*. **2004**, *43*, 449–488.

Schwertner, H. A.; Mosser, E. L. Comparison of lipid fatty acids on a concentration basis vs weight percentage basis in patients with and without coronary artery disease or diabetes. *Clin. Chem.* **1993**, *39*, 659–663.

Sek, L.; Porter, C. J. H.; Charman, W. N. Characterisation and quantification of medium chain and long chain triglycerides and their in vitro digestion products, by HPTLC coupled with in situ densitometric analysis. *J. Pharm. Biomed. Anal.* **2001**, *25*, 651–661.

Singh, A. K.; Jiang, Y. Quantitative chromatographic analysis of inositol phospholipids and related compounds. *J. Chromatogr. B Biomed. Appl.* **1995**, *671*, 255–280.

Skipski, V. P.; Peteron, R. F.; Sanders, J.; Barclay, M. Thin-layer chromatography of phospholipids using silica gel without calcium sulfate binder. *J. Lipid Res.* **1963**, *4*, 227–228.

Skipski, V. P.; Peterson, R. F.; Barclay, M. Quantitative analysis of phospholipids by thin-layer chromatography. *Biochem. J.* **1964**, *90*, 374–378.

Sommerer, D.; Suss, R.; Hammerschmidt, S.; Wirtz, H.; Arnold, K.; Schiller, J. Analysis of the phospholipid composition of bronchoalveolar lavage BAL fluid from man and minipig by MALDI-TOF mass spectrometry in combination with TLC. *J. Pharm. Biomed. Anal.* **2004**, *35*, 199–206.

St Angelo, A. J.; James, C. Jr. Analysis of lipids form cooked beef by thin-layer chromatography with flame ionization detector. *Lipids* **1993**, *70*, 613–665.

Steele, W.; Banks, W. Triglyceride distribution in hydrogenated milk fat and its effects on separation by thin layer chromatography. *Milchwissenschaft* **1994**, *49*, 372–376.

Tanamati, A.; Oliveira, C. C.; Visentainer, J. V.; Matsushita, M.; de Souza, N. E. Comparative study of total lipids in beef using chlorinated solvent and low-toxicity solvent methods. *J. Am. Oil Chem. Soc.* **2005**, *82*, 393–397.

Vaden, D. L.; Gohil, V. M.; Gu, Z.; Greenberg, M. L. Separation of yeast phospholipids using one-dimensional thin-layer chromatography. *Anal. Biochem.* **2005**, *338*, 162–164.

Valls, J. E.; Bello, R. A.; Kodaira, M. S. Semiquantitative analysis by thin-layer chromatography TLC of biogenic amines in dried, salted and canned fish products. *J. Food Qual.* **2002**, *25*, 165–176.

Vaskovsky, V. E.; Kostetsky, E. Y. Modified spray for the detection of phospholipids on thin-layer chromatograms. *J. Lipid Res.* **1968**, *9*, 396.

Vecchini, A.; Chiaradia, E.; Covalovo, S.; Binaglia, L. Quantitation of phospholipids on thin layer chromatographic plates using a desk-top scanner. *Mol. Cell. Biochem.* **1995**, *145*, 25–28.

Vioque, E.; Holman, R. T. Characterization of the ketodienes formed in the oxidation of linoleate by lipoxidase. *Arch. Biochem. Biophys.* **1962**, *99*, 522–528.

Wagner, H.; Hoerhammer, L.; Wolff, P. Thin layer chromatography of phosphatides and glycolipids. *Biochem. Z.* **1961**, *334*, 175–184.

Wang, W. Q.; Gustafson, A. One-dimensional thin-layer chromatographic separation of phospholipids and lysophospholipids from tissue lipid extracts. *J. Chromatogr. B* **1992**, *581*, 139–142.

Wang, Y.; Sternfeld, L.; Yang, F.; Rodriguez, J. A.; Ross, C.; Hayden, M. R.; Carriere, F.; Liu, G.; Hofer, W.; Schulz, I. Enhanced susceptibility to pancreatitis in severe hypertriglyceridaemic lipoprotein lipase-deficient mice and agonist-like function of pancreatic lipase in pancreatic cells. *Gut* **2009**, *58*, 422–430.

Weerheim, A. M.; Kolb, A. M.; Sturk, A.; Nieuwland, R. Phospholipid composition of cell-derived microparticles determined by one-dimensional high-performance thin-layer chromatography. *Anal. Biochem.* **2002**, *302*, 191–198.

White, T.; Bursten, S.; Federighi, D.; Lewis, R. A.; Nudelman, E. High-resolution separation and quantification of neutral lipid and phospholipid species in mammalian cells and sera by multi-one-dimensional thin-layer chromatography. *Anal. Biochem.* **1998**, *258*, 109–117.

Wilson, R.; Sargent, J. R. Chain separation of monounsaturated fatty acid methyl esters by argentation thin-layer chromatography. *J. Chromatogr. A* **2001**, *905*, 251–257.

Yu, Q. T.; Liu, B. N.; Zhang, J. Y.; Huang, Z. H. Location of methyl branchings in fatty acids: fatty acids in uropygial secretion of Shanghai duck by GC-MS of 4,4-dimethyloxazoline derivatives. *Lipids* **1988**, *23*, 804–810.

Yurkova, I.; Shadyro, O.; Kisel, M.; Brede, O.; Arnhold, J. Radiation-induced free-radical transformation of phospholipids: MALDI-TOF MS study. *Chem. Phys. Lipids* **2004**, *132*, 235–246.

Zalata, A. A.; Christophe, A. B.; Depuydt, E.; Schonjans, F.; Comhaire, F. H. The fatty acid composition of phospholipids of spermatozoa from infertile patients. *Mol. Hum. Reprod.* **1998**, *4*, 111–118.

Zemski Berry, K. A.; Murphy, R. C. Electrospray ionization tandem mass spectrometry of glycerophosphoethanolamine plasmalogen phospholipids. *J. Am. Soc. Mass Spectrom.* **2004**, *15*, 1499–1508.

Zhong, J. H.; Guo, Q. Y.; Ye, R. G.; Lindholm, B.; Wang, T. Phospholipids in dialysate and the peritoneal surface layer. *Adv. Perit. Dial.* **2000**, *16*, 36–41.

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