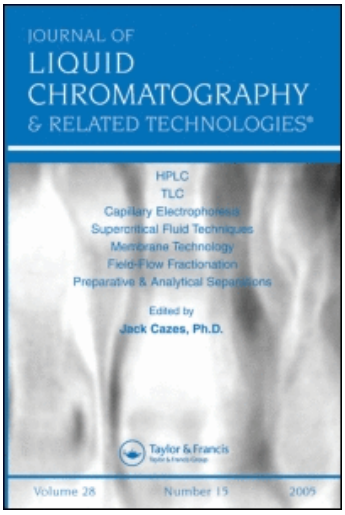

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Separation of Phospholipids by HPTLC – An Investigation of Important Parameters

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Abstract: Numerous chromatographic systems have been reported in the literature for the separation of phospholipids by High Performance Thin-Layer Chromatography. We have noticed that the reproducibility of analytical results of most of these methods is not satisfactory over longer periods of time, even when modern instruments are employed. No theory explaining the reasons for this problem currently exists. We have investigated several parameters of the chromatographic process, including chamber saturation, derivatization, plate activity, and batch to batch consistency of the plates. This paper provides a summary of results obtained in our laboratory over more than six years.

Separation of the phospholipids PA, PC, PE, PI, LPA, LPC, LPE, and LPI can be achieved on HPTLC silica gel 60 (Merck) with chloroform, methanol, water, ammonia 25% (60:34:4:2) as mobile phase. For reproducible results, the employed methodology must be strictly standardized. Most importantly, the developing chamber must be homogenously saturated for a specified time and the activity of the layer should be kept constant.

Keywords: High performance thin layer chromatography (HPTLC), Identification, LPA, LPC, LPE, LPI, PA, PC, PE, Phospholipids, PI, Quantitative analysis

INTRODUCTION

Thin-layer chromatography (TLC) and its high-performance version, HPTLC, is used extensively for lipid analysis and is an established and

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valuable tool for separation, tentative identification, and quantitative investigation of lipids from different classes. A comprehensive overview and discussion has been presented by Fried.[1] Most of the original papers published on the subject have a strong biological, biochemical, or clinical focus and generally report only briefly the chromatographic conditions that are useful to answer specific analytical questions.

Among the various lipid classes, phospholipids represent a very challenging one because they not only include six subgroups and their lyso-forms (Figure 1), but also occur naturally in complex mixtures with other polar and non-polar lipids.

Aside from their biochemical relevance as primary constituents of membranes, phospholipids are of particular value to the pharmaceutical and cosmetic industry. Therefore, analytical questions related to the quality of raw materials and products have become economically significant and of interest to researchers as well. On average, four papers on TLC/HPTLC of phospholipids have been published annually over the last decade.[2] Recently, these papers have focused on the use of modern equipment and report in greater detail how specific separations have been achieved.[3–5]

A review of the available literature reveals that most TLC separations of phospholipids are carried out on unmodified silica gel as stationary phase. Chloroform, methanol, water mixtures in various proportions without or with the addition of other organic solvents (e.g., isopropanol, acetone, ethyl acetate, etc.) or modifiers (e.g., ammonia, acetic acid, amines, potassium chloride, etc.) are reported as mobile phases. Due to the diversity of analytical questions and the complexity of phospholipid containing samples, it cannot be expected

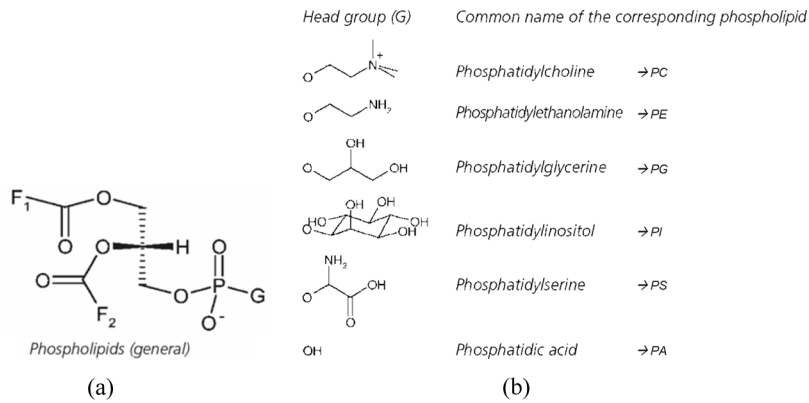


Figure 1. (a) General structure of phospholipids (F1, F2: fatty acid groups; G: head group); (b) one of the fatty acid groups is removed in lyso-forms.

that any single chromatographic system (combination of stationary and mobile phase) would provide a universal solution. Consequently, numerous methods are proposed, but there seems to be no underlying theory about why any given method achieves what it does. None of the reviewed publications addresses the possible effects that other parameters of the TLC process could have on the separation and whether those could offset changes in the principal chromatographic system. Even more puzzling to us was the fact that different publications report different R_F values for the same reference compounds (e.g., phosphatidylcholine (PC), phosphatidylethanolamine (PE), lysophophatidylcholine (LPC)) even when the chromatographic system is the same. Similar findings have been obtained in our laboratory, usually when we either tried to repeat analyses from the literature or repeat our own work over a period of time. This has led us to a systematic investigation of several HPTLC parameters and their effects on the separation of phospholipids. The primary focus of the work was on a standardized methodology to improve the predictability and reproducibility of an analysis. An integral part of the project was the work of C. Schärer and J. Hellwig, students at the University of Applied Sciences Northwestern Switzerland, in 2002 and 2004.[6,7]

This paper provides a summary of results obtained in our laboratory over more than six years. It should draw the attention of analysts towards experimental details, details that may be easily overlooked, yet may have very significant effects. We are not suggesting that our proposal will solve all problems associated with the analysis of phospholipids but, at least, it presents measures that will help to keep the experimental conditions under tight control.

EXPERIMENTAL

Materials

Reference substances for different classes of phospholipids were obtained from Sigma (Buchs, Switzerland), Fluka (Buchs, Switzerland) and Avanti Polar Lipids Inc. (Alabaster, AL).

HPTLC glass plates coated with silica gel 60 F_{254} were manufactured by Merck (Darmstadt, Germany). Solvents of p.a. grade and general chemicals were purchased from Acros (Gent, Belgium), Fluka (Buchs, Switzerland) or Merck. Chromatographic equipment (Automatic TLC Sampler 4, Twin Trough Chamber, ADC 2 Automatic Developing Chamber with humidity control unit, Immersion Device, Plate Heater, DigiStore2 digital documentation system, TLC Scanner 3, winCATS

1.4.3 software, and VideoScan 1.02 software) was made by Camag (Muttenz, Switzerland).

Preparation of Samples

Solutions of individual phospholipids were prepared at a concentration of 0.1 mg/mL in chloroform, methanol (2:1): Phosphatidic acid (PA), Phosphatidylethanolamine (PE), Phosphatidylinositol (PI), Phosphatidylcholine (PC), Lysophosphatidic acid (LPA), Lysophosphatidylethanolamine (LPE), Lysophosphatidylinositol (LPI), and Lysophosphatidylcholine (LPC).

Preparation and Use of Derivatizing Reagents

Zirconium (IV) oxide chloride reagent was prepared by dissolving 5 g of $ZrOCl_2$ in 250 mL of methanol.[8] The developed plate is immersed into the reagent for 1 s. Then the plate is heated at 180°C for 20 min using a plate heater.

Ammonium molybdate reagent was prepared by dissolving 1 g of ammonium molybdate in 10 mL of water. To the solution, 3 mL of HCl 25% and 5 mL of perchloric acid 60% were added. The final volume of the mixture was adjusted to 200 mL with acetone.[9] The developed plate is immersed into the reagent for 2 s. Then, the plate is heated at 110°C for 20 min using a plate heater.

Antimony (III) chloride reagent was prepared by dissolving 1 g of $SbCl_3$ in 50 mL of chloroform. To the solution, a mixture of 45 mL of methanol and 5 mL of sulfuric acid 98% was added.[10] The developed plate is immersed into the reagent for 1 s. Then, the plate is heated at 120°C for 20 min using a plate heater.

Copper (II) sulfate reagent was prepared by dissolving 50 g of anhydrous copper (II) sulfate in 300 mL of deionized water and adding 40 g of ortho-phosphoric acid 85%. This mixture was filled up to 500 mL with deionized water.[6] The developed plate is immersed into the reagent for 2 s. Then, the plate is heated at 175°C for 20 min using a plate heater.

Modified copper sulfate reagent was prepared by dissolving 20 g of copper (II) sulfate pentahydrate in 200 mL of cooled methanol. Under cooling with ice, 8 mL of sulfuric acid 98% and 8 mL of ortho-phosphoric acid 85% were added to the solution. The temperature of the mixture should not increase above 20°C. The developed plate was immersed in the reagent for 6 s, and then dried with cold air from a hair dryer for 30 sec. Finally, the plate was heated at 140°C for 30 min using a plate heater.

Rhodamine B reagent was prepared by dissolving 50 mg of rhodamine B in 200 mL of deionized water.[11] The developed plate is immersed into the reagent for 2 s. Then, the plate is dried with cold air from a hairdryer.

Chromatography and Evaluation

The general SOP for HPTLC as previously published[12] was followed. Sample volumes of 2–10 μL were applied as 8 mm bands using the spray-on technique. HPTLC plates were developed with chloroform, methanol, water, ammonia 25% (60:34:4:2) over a distance of 70 mm from the lower edge of the plate using an ADC 2 Automatic Developing Chamber in saturated mode. The twin trough chamber of the ADC 2 was fitted with a filter paper in the rear trough. The front trough was charged with 10 mL developing solvent, the rear trough with 25 mL solvent. Chamber saturation time was 20 min. Inside of the ADC 2, prior to development, the plate was conditioned to 47% relative humidity (RH) for 10 min using a saturated solution of KSCN in water.[13]

Except for the initial experiments, the developed plates were derivatized with modified copper sulfate reagent. A digital image of the plate was captured under white light.

The quantitative evaluation of individual phospholipids was performed by scanning densitometry in absorption mode at 360 nm (deuterium lamp), 420 or 720 nm (tungsten lamp), or by video densitometry of images taken under white light.

RESULTS AND DISCUSSION

Selection of the Chromatographic System

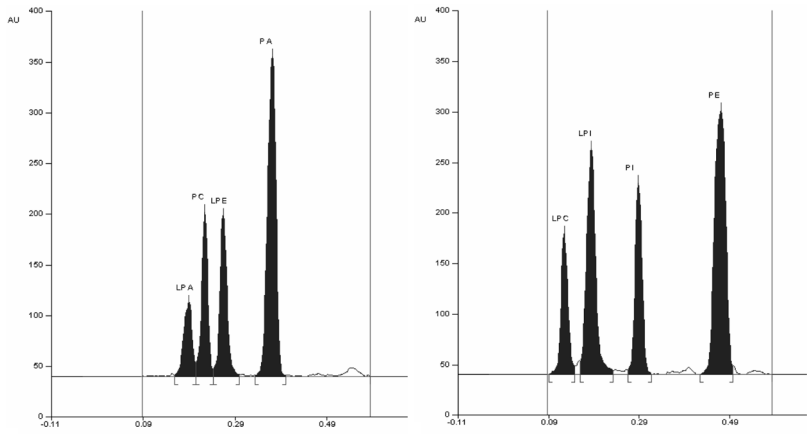
The starting point of our work was a comparison of several chromatographic systems from the literature (Table 1) applying a standardized HPTLC methodology. For chromatogram development, a standard saturated twin trough chamber with saturation time of 20 min was used. The results of the analysis were compared with respect to the separation of four phospholipids and their lyso-forms: PA/LPA, PI/LPI, PC/LPC, PE/LPE.

Chloroform, methanol, water, ammonia 25% (65:30:4:2) as mobile phase overall gave the best performance. In deviation from the HPTLC SOP[12] the developing distance was increased to 80 mm from the lower edge of plate because the R_F values of the separated compounds were below 0.50 and the actual distance between the bands was small. Except for LPI and LPA, all substances have different R_F values under these

Table 1. Investigated chromatographic systems for the separation of phospholipids

Stationary phase	Mobile phase	Reference
HPTLC Si 60 F_{254}	Chloroform, methanol, water, ammonia 25% (65:30:4:2)	[14]
HPTLC Si 60 F_{254}	Chloroform, methanol, water (65:25:4)	[15]
HPTLC Si 60 F_{254}	Chloroform, methanol, acetic acid, water (25:45:4:2)	[15]
HPTLC Si 60 F_{254}	Chloroform, methanol, water (35:25:4)	[16]
HPTLC Si 60 F_{254} Impregnated with boric acid	Chloroform, ethanol, triethylamine, water (30:35:6:35)	[17]
HPTLC Si 60 F_{254} Impregnated with ammonium sulfate	Chloroform, methanol, acetic acid, acetone, water (40:25:7:4:2)	[18]

conditions, as seen in Figure 2. When grouped into two mixtures of standards, so that LPI and LPA are not in the same mixture, it is possible to qualitatively and quantitatively determine the individual compounds following derivatization with copper sulfate reagent.



(a) (b)

Figure 2. Separation of 8 phospholipids as part of two mixtures on different tracks. Note: LPI and LPA have the same R_F values. Chromatography: HPTLC silica gel 60 F_{254}; chloroform, methanol, water, ammonia 25% (65:30:4:2); densitometry at 420 nm after derivatization with copper sulfate reagent.

Table 2. Working ranges for quantitative determination of phospholipids

	Working range (ng/zone)	LOD (ng)	LOQ (ng)	RSD of calibration curve (%)
LPC	170–216	25	104	7.1
LPA	260–558	25	78	1.71
PI	168–504	32	84	1.58
PE	340–1020	43	190	2.10
LPI	80–200	24	99	1.52
PC	206–490	89	373	2.69
LPE	170–408	82	353	2.73
PA	200–480	32	93	1.29

Table 2 lists working ranges for the investigated substances based on five point calibration and polynomial regression.

It was found that the concentration of the ammonia solution used to prepare the mobile phase and the homogeneity of the chamber saturation are of great importance for the reproducibility of results.

Optimization of Detection

None of the phospholipids has a chromophore that could be utilized for detection on the HPTLC plate. This makes a chemical derivatization necessary for evaluation. Various derivatization reagents are described in the literature. They typically include a transition metal and depend on heat for completion of the reaction. Phospholipids are transformed into colored derivatives which can be evaluated under white light. Often, these derivatives also show fluorescence when excited with UV 366 nm. Other reagents such as rhodamine B form adducts with phospholipids and do not require a heating step. Figure 3 provides a comparison.

The derivatization experiments led to three conclusions: (1) not all phospholipids react equally well with a given reagent; there are differences in the required reaction times for the individual substances, and equal amounts of substances do not necessarily produce derivatives of the same intensity. (2) Except for rhodamine B, all other reagents from the literature offer comparable sensitivity for quantitation. Evaluation in absorption mode gave, generally, less baseline noise than fluorescence measurement. Rhodamine B reagent visualizes different compounds in absorption and fluorescence mode, which could make detection more selective. (3) For reproducible results across the HPTLC plate, heating must be performed in a homogenous way. Because an initial slight deformation/warping of the plate and the resulting irregularities in heat

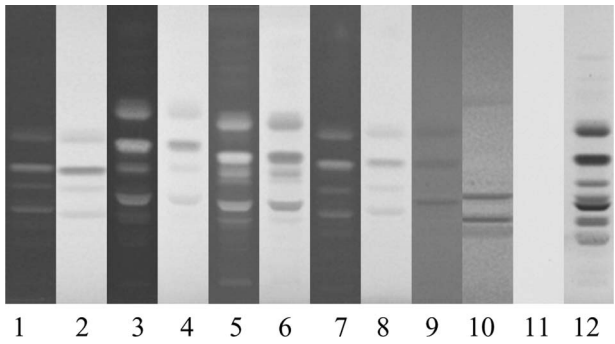


Figure 3. Post-chromatographic derivatization of phospholipids; chromatography as in Figure 2. 1,2: zirconium (IV) oxide chloride reagent; 3,4: ammonium molybdate reagent; 5,6: antimony (III) chloride reagent; 7,8: copper (II) sulfate reagent; 9,10: rhodamine B reagent; 11: blank; 12: modified copper (II) reagent. Odd numbered tracks, (except 11) under UV 366 nm, even numbered tracks under white light. Note: images of tracks have been taken from different plates.

transfer cannot be avoided when placing a plate onto a hot plate heater (or into a hot oven) for derivatization, it is important that enough time is allowed for completion of the heat induced reaction. A short heating period at higher temperature is less suitable than a lower temperature combined with longer heating time. For best results, we propose the use of a modified copper sulfate reagent containing both sulfuric acid and phosphoric acid, extended immersion time for thorough transfer of the reagent into the layer, and a longer heating time (30 min) at 140°C (Figure 9).

Optimization of Chamber Parameters

During experiments in a regular saturated twin trough chamber, we observed that reproducibility of chromatography was not good. Not only did the R_F values of the phospholipids vary, in general, but also the relative positions of individual compounds shifted. Therefore, we employed an ADC 2 Automatic Developing Chamber to manage the principal parameters of the development process. This device allows modifying and controlling the activity of the stationary phase prior to development, the degree of chamber saturation, the developing distance, and, finally, controling the drying process after chromatography. Initially, the established chromatographic system (HPTLC silica gel 60 F_{254}; chloroform, methanol, water, ammonia 25% (65:30:4:2);

copper sulfate reagent) was employed with 20 min chamber saturation. The developing distance from lower edge of plate was set back to the standard value of 70 mm. Surprisingly, it was impossible to achieve in the ADC 2 Automatic Developing Chamber the same separation as shown in Figure 2. Generally, methods can be transferred to an ADC 2 without problems, but the phospholipids reacted noticeably differently to this change in chamber. This prompted us to re-evaluate the composition of the mobile phase. Chloroform, methanol, water, ammonia 25% (60:35:4:2) was selected because this combination resolved all eight phospholipids (Figure 4).

If full chamber saturation is not established prior to chromatography, the separation is not uniform across the plate. As seen in Figure 5, LPC and LPI are separated on track 1 but not on track 14 (lower arrows), while on track 1 LPE and PI co-migrate but on track 14 these compounds begin to separate (upper arrows).

The separation is strongly dependent, also, on the activity of the stationary phase; it is best if the plate is conditioned for 10 min to a relative humidity of 47% (Figure 6).

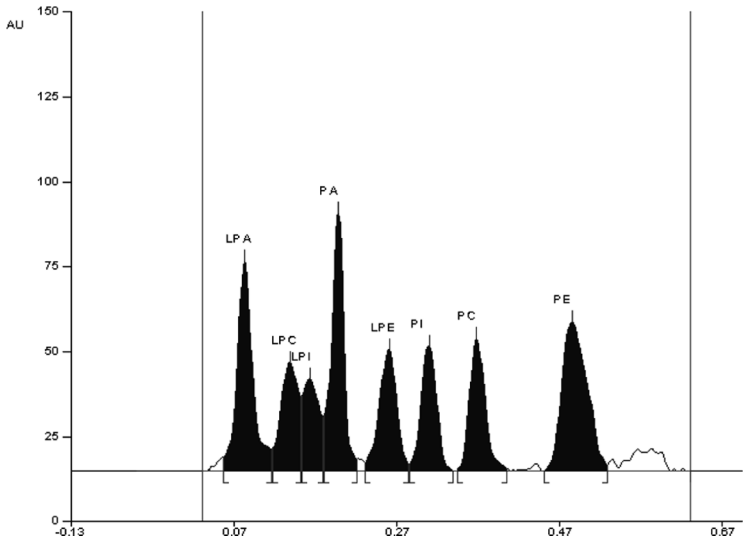


Figure 4. Separation of eight phospholipids on the same track in the ADC 2 Automatic Developing Chamber; chromatography: HPTLC silica gel 60 F_{254}; chloroform, methanol, water, ammonia 25% (60:35:4:2); developing distance from lower edge 70 mm, densitometry at 720 nm following derivatization with copper sulfate reagent.

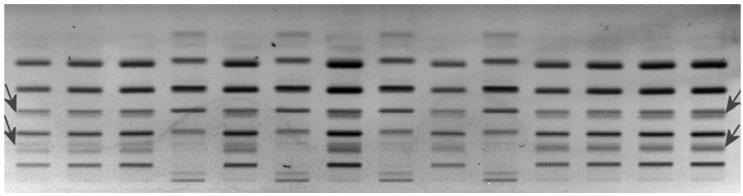


Figure 5. Effects of chamber saturation on the separation of eight phospholipids, chromatography as in Figure 4, except plate saturation time 10 min.

Other Parameters

Particularly for quantitative determinations, it is a good practice to pre-wash the plate to remove any contamination from the stationary phase and to improve the signal-to-noise ratio. In our investigation, we compared the performance of one untreated plate with that of a plate pre-washed with methanol, dichloromethane (1:1), and dried at 120°C for 20 min, one plate pre-washed with methanol and dried at 120°C for 20 min, and one plate that was not pre-washed but heated at 120°C for 60 min. All other parameters, including adjustment of activity to a relative humidity of 47%, were kept constant.

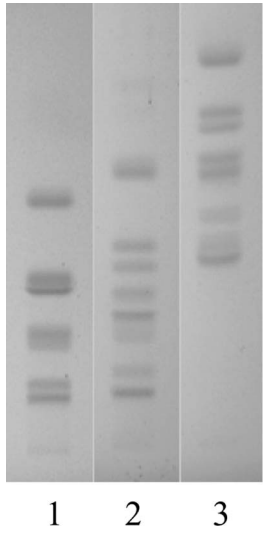


Figure 6. Effects of plate activity on the separation of 8 phospholipids, chromatography as in Figure 4. Plates are conditioned for 10 min to 1: molecular sieve (RH < 5%); 2: saturated KSCN solution (RH 47%); 3: saturated NaCl solution (RH 75%). Note: individual tracks are taken from different plates.

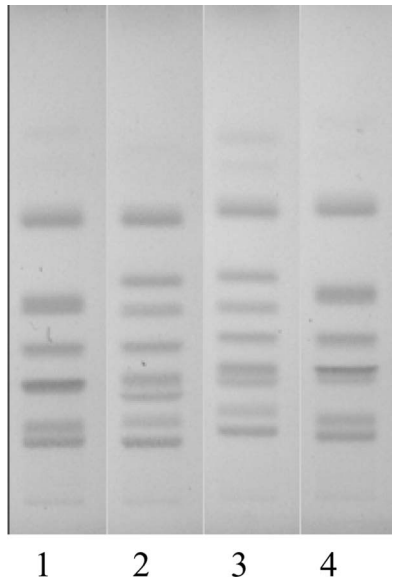


Figure 7. Effects of pre-washing/drying on the separation of eight phospholipids, chromatography as in Figure 4. 1: pre-washed with methanol, dichloromethane (1:1), dried at 120°C for 20 min; 2: untreated plate; 3: not pre-washed, dried at 120°C for 60 min; 4 pre-washed with methanol, dried at 120°C for 20 min. *Note*: individual tracks are taken from different plates.

Figure 7 illustrates the finding that only the untreated plate (as it comes out of the box) gives the desired results. Even though the plate is conditioned with a fixed relative humidity prior to chromatography, an initial heating (activation) affects the chromatographic result. It is evident that the pre-washing affects the separation of LPC and LPI. All three results are exceptions to the common understanding that (1) during 20 min heating at 120°C, the pre-washing solvent is completely removed and (2) the effect of the conditioning with a fixed relative humidity is independent of the plate activity prior to the conditioning step.

Up to this point, all experiments in the ADC 2 Automatic Developing Chamber have been conducted on plates of the batch OB562573, while the earlier manual experiments utilized plates of the batches OB259812, OB143830, and OB337834 in combination with a different mobile phase. In January of 2007, we switched to batch HX745979 and noticed that chromatography had changed significantly, even though all other parameters were kept constant. This initiated an investigation of the batch to batch consistency of the plates (Figure 8), which revealed that no two batches give exactly the same result.

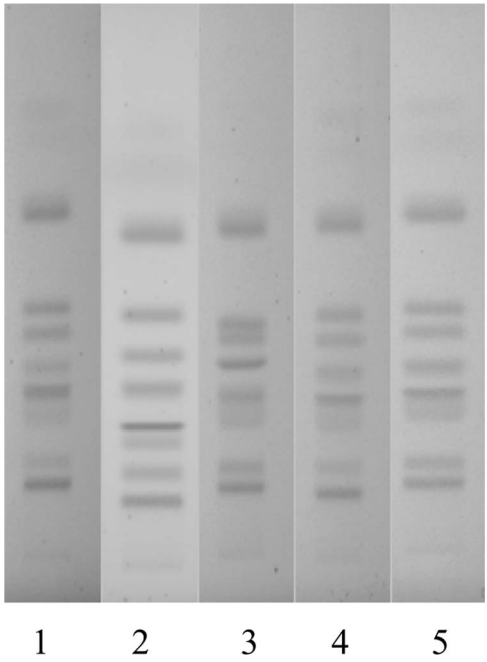


Figure 8. Comparison the separation obtained on HPTLC plates from 5 different batches. Chromatography as in Figure 4. 1: OB562573; 2: HX745979; 3: HX612152; 4: OB680980; 5: HX693630.

As long as no significant change in the separation results, this fact may be considered unimportant. Unfortunately, at that time the current batch of plates produced results which were not comparable to previous results. It was necessary to, again, slightly adjust the mobile phase by removing one part more methanol resulting in: chloroform, methanol, water, ammonia 25% (60:34:4:2). In some experiments, the developing distance was reduced to 60 mm from the lower edge of plate in order to obtain sharper zones. This was done in view of a possible quantitative evaluation (Figure 9). The use of a modified copper acetate reagent (see above) for derivatization provided excellent reproducibility across the plate. Relative standard deviation of peak area/height for 13 tracks was 1.5/1.8% for PC. All investigated phospholipids give a similar response. For best results (lowest signal to noise ratio), quantitative evaluation by scanning densitometry should be performed in absorption mode at 360 nm using a deuterium lamp. An alternative is video-densitometric evaluation of the image of the derivatized plate. A prerequisite is that the image is captured with a suitable documentation system, which is able to correct any inhomogeneity of the illumination as well as irregularities of the plate background.

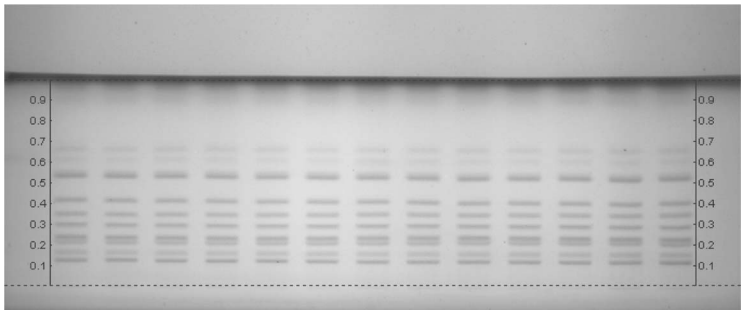


Figure 9. Separation of eight phospholipids on HPTLC plates of batch HX745979 with water: chloroform, methanol, water, ammonia 25% (60:34:4:2); 60 mm from lower edge of plate; modified copper sulfate reagent.

CONCLUSION

Separation of the phospholipids PA, PC, PE, PI, LPA, LPC, LPE, and LPI can be achieved on HPTLC silica gel 60, batch HX745979 (Merck) with chloroform, methanol, water, ammonia 25% (60:34:4:2) as mobile phase. For reproducible results, the employed methodology must be strictly standardized. Most importantly, the developing chamber must be homogenously saturated for a specified time (e.g., 20 min) and the activity of the layer should be kept constant (e.g., adjusted to 47% relative humidity). An automatic developing chamber with humidity control is ideally suited for achieving both. Differences in the performance of the stationary phase (HPTLC silica gel 60) from batch to batch must be anticipated. These differences can usually be offset by small changes to the composition of the mobile phase keeping all other parameters constant. Such changes must be made on a trial and error basis. For qualitative and quantitative evaluation of separated phospholipids, following derivatization with a modified copper sulfate reagent, either scanning densitometry at 360 nm in absorption mode or image evaluation by video densitometry can be employed. The necessary quality of the images required by the latter can be achieved by a suitable digital documentation system such as the DigiStore2.

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