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Review

Derivatization of phospholipids

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Abstract

Phospholipids are major components of biological membranes. Without chemical derivatization, it is difficult to identify and quantitate phospholipids in biological samples. Chemical derivatization can improve both the selectivity and sensitivity of the analytes. This paper gives a full review, through March, 2002, of derivatization methods used for phospholipids in HPLC, CE and GC as well as the spray reagent used for TLC in the early days.

Keywords: Reviews; Derivatization; Phospholipids

Contents

1. Introduction

Phospholipids are lipids that contain phosphoric residues and are thus ampiphilic molecules possessing polar head groups and non-polar lipid chains. As ampiphiles, phospholipids self-associate in aqueous solutions at concentrations above their critical micelle concentration [1]. They are major lipid components of biological membranes. Existing as many different kinds of compounds, phospholipids play an important role in the signal transduction

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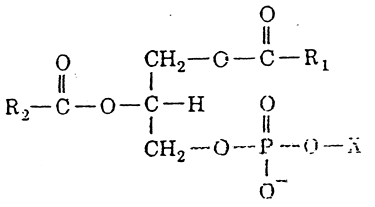
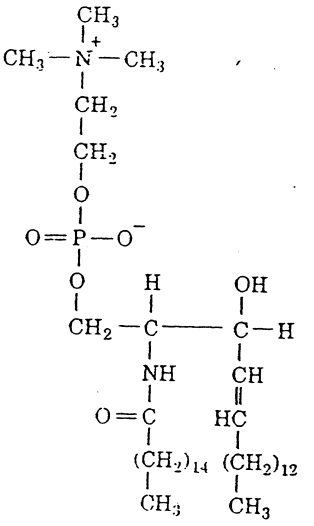
process. An examination of chemical composition of mammalian cell membranes reveals that over 90% of their mass (dry weight) is comprised of proteins and phospholipids [2].

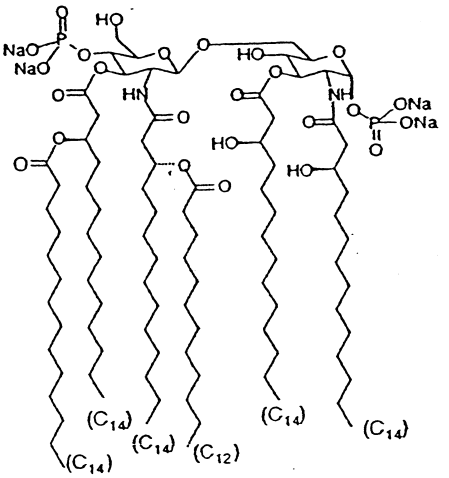
There are two major classes of phospholipids: one is phosphoglyceride (or glycerophospholipid) that has a glycerol backbone; the other is sphingosine-based phospholipid, sphingomyelin. Phosphoglycerides consist of glycerol-3-phosphate, which is esterified at its C-1 and C-2 positions to fatty acids and at its phosphoryl group to another group X, to form the class of compounds (Fig. 1a) [3]. The X group is typically choline, ethanolamine, serine, inositol, etc. The corresponding phosphoglycerides are named phosphatidylcholine (PC) and phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), etc., as shown in Table 1. Some phospholipids that are present in very low concentrations are called minor phospholipids, such as platelet activating factor (PAF), phosphatidic acid, sphingosine-1-phosphate and so on. PAF denotes a unique autacoid class of 2-acetylated phospholipids (1-*O*-alkyl-2-acetyl-glycero-3-phosphocholine), which has the property of platelet activation [2]. Sphingomyelins are ceramides bearing either a phosphocholine or a phosphoethanolamine group (Fig. 1b).



(a) Glycerophospholipid



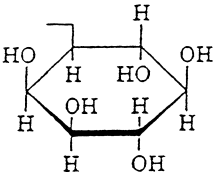
(b) A sphingomyelin



(c) E coli lipid A

Fig. 1. Structures of phospholipids. (a) Glycerophospholipid, (b) a sphingomyelin, (c) *E. coli* lipid A.

Table 1
The common classes of glycerophospholipids

Name of X–OH	Formula of –X	Name of phospholipid
Water	–H	Phosphatidic acid
Choline	$-CH_2CH_2N(CH_3)_3^+$	Phosphatidylcholine
Ethanolamine	$-CH_2CH_2NH_3^+$	Phosphatidylethanolamine
Glycerol	$-CH_2CH(OH)CH_2OH$	Phosphatidylglycerol
myo-Inositol		Phosphatidylinositol
Serine	$-CH_2CH(NH_3^+)COO^-$	Phosphatidylserine

Lipid A (Fig. 1c), the hydrophobic anchor of lipopolysaccharide (LPS; endotoxin) from the gram-negative bacteria outer membrane, is an important class of phospholipid that is related to sepsis. It has a disaccharide backbone attached with several fatty acid chains. Lipid A triggers the biosynthesis of diverse mediators of inflammation and is suspected to be the causative agent of septic shock syndrome [4–6]. Lipid A exists in two forms: di-phosphoryl lipid A and monophosphoryl lipid A.

All of the phospholipids consist of long-chain fatty acids and phosphoryl groups. Since the fatty chains can vary in length and degree of unsaturation, each phospholipid class has numerous molecular species with different chemical and biological properties. Identification and quantitation of phospholipids in biological samples has been of great interest [2].

Lack of chromophores has been a problem for identification and quantification of phospholipids. Ultraviolet (UV) detection is usually used in the 200–214 nm range, due to the presence of an unsaturated functional group, such as carbonyl, carboxyl and phosphate. Mass spectrometry (MS), the most sensitive and specific detection technique, is relatively expensive and less widely used. Chemical derivatization, which gives detection of the analytes not only selectively but also sensitively, is very useful in phospholipid analysis. By reacting with certain reagents, phospholipids are transformed to derivatives that can absorb UV light or emit fluorescence (FL). In this review, we cover phospholipid derivatization in high-performance liquid chromatography (HPLC), capillary electrophoresis (CE) and gas chromatography (GC) analysis, with UV, FL and MS detection. A brief review of intact phospholipids analysis is also given.

2. Separation and detection methods of underivatized phospholipids

Thin layer chromatography (TLC), HPLC and CE are common techniques used for separation of phospholipids. For TLC, densitometry is usually employed for quantitative determination. UV, LSD and MS have also been coupled to HPLC or CE. In this section, a brief overview of these techniques is given.

2.1. TLC with colorimetric (densitometry) detection

One- or two-dimensional TLC has often been used to separate phospholipid extracts since the 1960s. Usually, the subclasses obtained by TLC are subject to HPLC for further analysis. After the separation, the phospholipid components are visualized and quantitated through the use of spray reagents. By phosphorus and char reactions, or spraying other detection reagents like ninhydrin, Dragendorff [7–9] or 6-*p*-toluidineo-2-naphthalenesulfonic acid (TNS) [10], phosphorus-containing lipids are detected on the TLC plate. Dittmer and Lester also developed a

spray reagent to react specifically with phospholipids [11]. TLC has been routinely used for isolation and identification of phospholipids from biological samples. However, further analysis by HPLC and/or MS is required for specific molecules analysis because of its low resolution and poor reproducibility.

2.2. HPLC and CE with UV, LSD and MS detection

Compared to TLC, HPLC can provide higher selectivity and efficiency, and it can separate each phospholipid subclass into more species. Jungalwala et al. first used HPLC to separate PE and PS with derivatization on the primary amino group [11]. Right after that, Jungalwala et al. [12] developed a HPLC method for PC and sphingomyelin without derivatization, which was complementary to the first approach. Since then, numerous HPLC methods have been developed and applied to phospholipid analysis.

For intact phospholipids, UV detection was mostly used at 200–214 nm. The sensitivity was dependent on the structures of the phospholipids and the choice of mobile phase. With UV detection, two different mobile phases are applied most frequently: *n*-hexane–2-propanol–water and acetonitrile–methanol–water [13].

A universal detector, the light-scattering mass detector (LSD) has also been used [14,15]. Van der Meeren et al. [14] developed a simple and rapid separation and quantification of soybean phospholipids, using HPLC coupled with LSD. The non-volatile phospholipids remained as particles after evaporation of the nebulised mobile phase. Light scattered by this particle cloud was detected by a photomultiplier. Quantification of phospholipids was obtained using a calibration curve. LSD has not been widely accepted as yet because of the poor linearity of its calibration curve, non-robustness compared to UV detection, the need for volatile additives and validation problems.

With fast developments in recent years, MS has been widely used by chemists, pathologists, clinicians and many others. It not only offers the capability to elucidate the structure of the analytes, but also has some resolving power by tandem MS (MS–MS). Phospholipids have been characterized by various MS modes, such as MALDI [16], ESI [17] and FAB [18–20]. In some cases, MS was coupled with chromatography [21,22] or CE [23]. Though being used more widely than before, MS is still an expensive technique.

Indirect detection is a choice when other detection techniques cannot be applied. Recently, an indirect-UV CE method was developed for the analysis of LPS, which also lacks optically active groups [24]. LPS was quantified with a limit of detection (LOD) down to 35 p*M* by using a strongly UV-active electrophoresis buffer. Fig. 2 shows the electropherograms of LPS from *Salmonella minnesota* and *E. coli*, where LPS appeared as negative peaks.

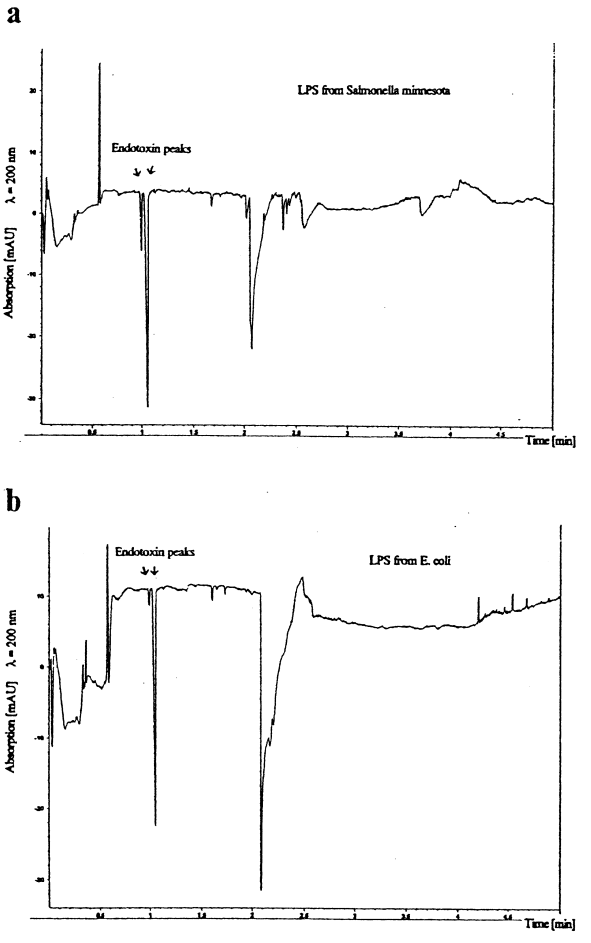


Fig. 2. Analysis of LPS using indirect UV detection. (a) LPS from *E. coli*. (b) LPS from *S. minnesota*. Reproduced with permission from Ref. [24].

Recently, CE of phospholipids with indirect photometric detection (IPD) was also reported [25].

In summary, those methods discussed above are either insensitive or expensive. An effective method of analysis is to derivatize the analytes prior to analysis. This has been widely used in many fields, such as analyses of amino acids, carbohydrates, etc. In the mid-1970s, people began to derivatize aminophospholipids for UV detection [11]. Since then, various reagents and methods have been developed for phospholipid analyses.

3. Separation and detection of derivatized phospholipids

3.1. Derivatizations in HPLC and CE

Most phospholipid analyses are carried out by HPLC. CE is relatively less widely used. In this section, we discuss derivatizations for improved sensitivity by UV and FL detection in HPLC and CE analyses. To derivatize phospholipids, they ought to have a chemically active group. For aminophospholipids such as PE and PS, the amino group is usually the derivatization site. For those phospholipids having no amino group or other active group, a hydroxy group is generated by enzymatic or chemical hydrolysis, especially for glycerophospholipids. We discuss in this section these two categories, phospholipids with amino groups and derivatization after hydrolysis, as well as some other miscellaneous derivatizations.

3.1.1. Phospholipids with amino groups

A sensitive HPLC–UV method was developed by Jungalwala et al. [11] for analysis of ethanolamine and serine-containing phospholipids in the form of biphenylcarbonyl derivatives at 280 nm. The limit of detection was ~10–13 pmol. Later on, Chen and co-workers derivatized aminophospholipids with DNS-chloride (1-dimethylaminonaphthalene-5-sulfonyl chloride) [26] and then succinimidyl 2-naphthoxyacetate [27] with FL detection. Fig. 3 shows the chromatograms of phospholipids as DNS and succinimidyl 2-naphthoxyacetate derivatives. Both of these methods had detection limits in the pmol range, and the latter one offered a higher speed

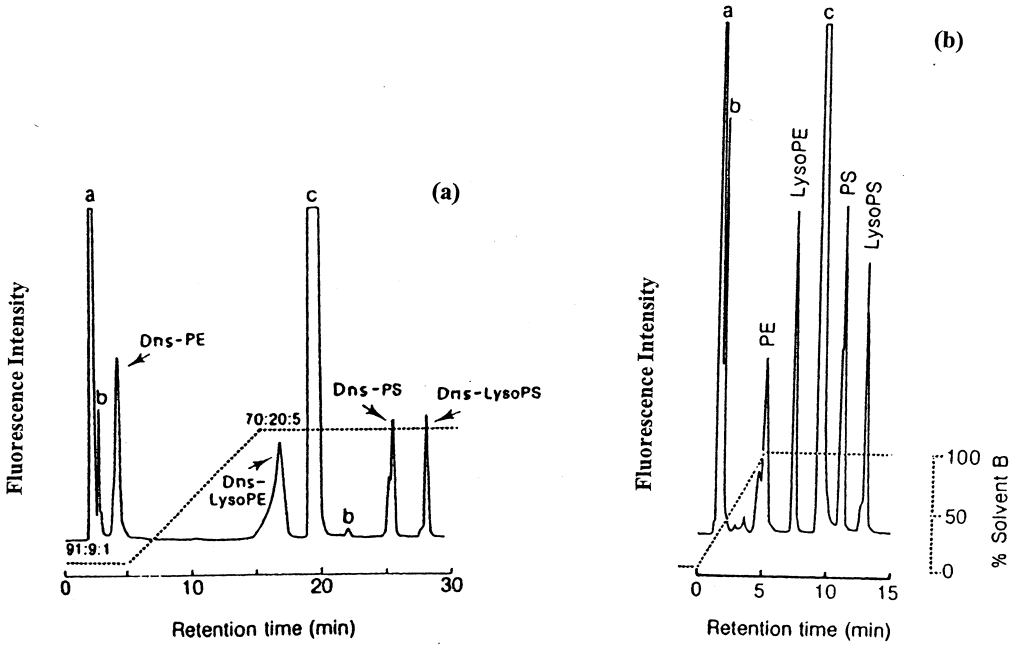


Fig. 3. Chromatograms of (a) DNS derivatives and (b) succinimidyl 2-naphthoxyacetate derivatives of phospholipids. Reproduced with permission from Refs. [26,27].

in the reaction and separation elution. Kaneko et al. [28] reported the application of 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) and 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) as pre-column FL labeling reagents for aminophospholipid analysis. NBD-F had advantages over NBD-Cl in the shorter reaction times and lower reaction amounts. With these two labeling reagents, PE, PS, lysoPE and lysoPS were derivatized within a short reaction time and the lack of interference from by-product peaks was observed. 4-Fluoro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-F) also found applications in the fluorimetric assay of phospholipase A acting on biomembrane phospholipids [29].

Phenylisothiocyanate (PITC) is a common derivatization reagent for amino acids and proteins. It reacts with primary and secondary amino groups to form stable phenylthiocarbamyl derivatives. For certain aminophospholipids, PE and PS, it also worked well, and it has been applied to analyze PE and PS, as well as proteins in lipid-rich samples. Stark and Johansson described the protocols for the entire process [30].

A fluorescent reagent, *ortho*-phthaldialdehyde (OPA) was used to derivatize sphingolipid metabolite sphingosine-1-phosphate (SPP) and other sphingoid base 1-phosphates, because of its specific reaction with primary amino groups [31–33]. SPP has an amino group at its C-2 position. After a two-step lipid extraction, SPP and dihydroSPP were transformed to fluorescent isoindole derivatives by OPA and then separated by HPLC. Quantification of SPP in the picomolar range was reported even in complex biological systems [33].

Abidi et al. [34] studied the HPLC retention behavior of several FL labeled phosphatidylethanolamine compounds in an ammonium acetate buffer. The labeled PLs included *N*-(rhodamine-B-sulfonyl)-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-PE, *N*-(5-fluoresceinthiocarbamoyl) (FL)-PE, *N*-(1-pyrenesulfonyl)-PE, and *N*-(5-dimethylaminonaphthalene-1-sulfonyl)-PE.

Recently, Le Zhang et al. [35] developed a micellar electrokinetic capillary chromatography (MECC) method with laser-induced fluorescence (LIF) detection for the analysis of amino-containing phospholipids, PE, PS, lysoPE and lysoPS. A fluorogenic dye, 3-(2-furoyl) quinoline-2-carboxaldehyde (FQ) and a fluorescence reagent, 4-fluoro-7-nitrobenzofurazan were tested. FQ-labeled phospholipids gave good responses for PE, PS, lysoPE and lysoPS, while with 4-fluoro-7-nitrobenzofurazan, FL signals were obtained only for PE and lysoPE. As shown in Fig. 4, FQ labeled lipids were completely separated with a methyl-β-cyclodextrin (CD)-modified MECC system. The detection limits ranged from 0.18 to 1.1 fg and was four- to five-orders of magnitude superior to CE–IPD [25].

3.1.2. Derivatizations after phospholipid hydrolysis

Out of the many cases of phospholipid analysis, a large number of derivatizations were performed after hydrolysis of phospholipids, especially for glycerophospholipids. After enzyme or chemical hydrolysis, glycerophospholipids are converted to diacylglycerols (DAGs) (Fig. 5), which are then acylated and esterified with UV absorbing or fluorescence chromophores on the free OH groups. This strategy can be applied for either separation or quantitation for phospholipids.

The formation and separation of UV and FL derivatives of DAG can be traced back to the 1980s. Batley et al. [36] developed a sensitive HPLC procedure for the separation of diglyceride *p*-nitrobenzoates with UV detection at 254 nm, which permitted complete analysis of phospholipids present

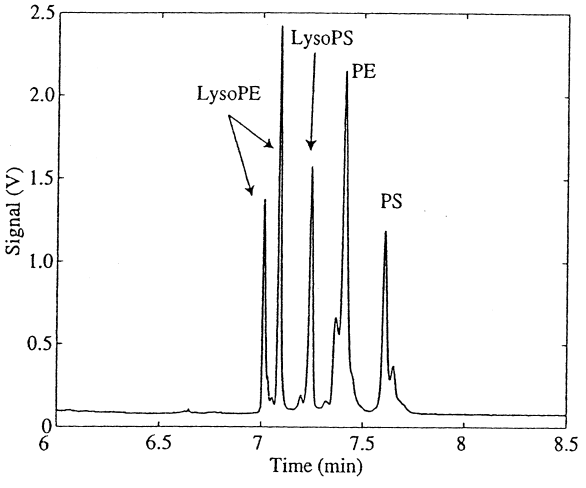


Fig. 4. Electropherogram of FQ labeled lipids obtained in 10 m*M* borax with 35 m*M* SDC and 7.5 m*M* methyl-β-CD. Reproduced with permission from Ref. [35].

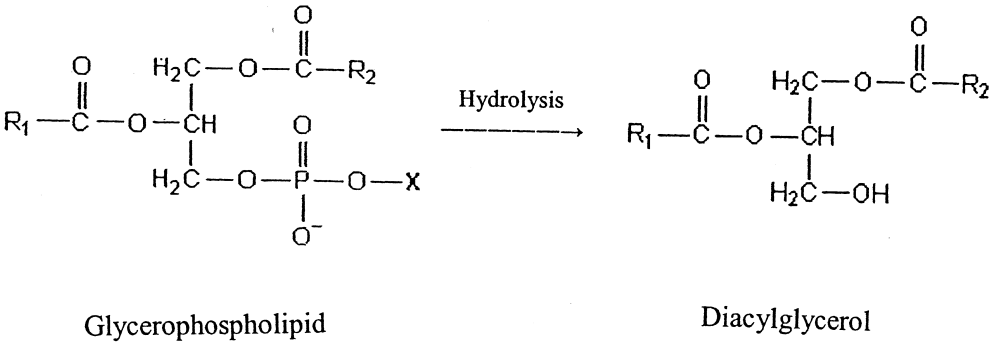


Fig. 5. Hydrolysis scheme of glycerophospholipid.

in the outer membranes of gram-negative bacteria. UV-absorbing methoxybenzoate [37] and dinitrobenzyl [38,39] derivatives were utilized for analysis of phospholipid molecular species. Blank et al. [40] reported another quantitative analysis of subclasses and molecular species within each subclass of glycerophosphatides. In all the cases, after phospholipase C hydrolysis, DAGs were transformed to benzoates and purified (or separated) by TLC. Each subclass was scraped off and extracted. The molecular species were then separated by HPLC with UV or FL detection. Clejan [41] recently described an entire separation procedure for DAG and phospholipid classes after derivatization by dinitro-benzoylation. 1-Anthroyl nitrile was also employed to react with DAGs in the presence of quinuclidine or 4-dimethylaminopyridine, forming a stable adduct for UV detection at 254 nm [42].

FL offers a much higher sensitivity than UV detection. By HPLC, Krueger et al. [43] separated and quantitated fluorescent naphthylisocyanate derivatives of diacylglycerols obtained from rat liver microsome phosphatidylcholine. DNS-ethanolamine phosphate is capable of reacting with DAG to form the FL phospholipid, DNS-phosphatidylethanolamine [44], which can be synthesized by reacting PE directly with DNS-Cl as described by Chen et al. [26]. This approach can also be monitored by TLC, as DNS derivatives emit FL light at visible wavelengths.

Another sensitive analysis for DAG was developed using naproxen chloride in the presence of 4-dimethylaminopyridine [45]. The high UV absorption coefficient of naproxen derivatives at 230 nm enable the quantitation in the picomole range. A 10-fold increase of sensitivity was achieved with FL detection because of the fluorescence properties of the adducts. Quantification of phospholipids in the femtomole level was achieved by Takamura and Kito [46] in the form of anthroyl DAG derivatives, with FL detection with excitation at 360 nm and emission at 460 nm. They applied this technique to analyze a biological phospholipid sample, Chinese hamster V79-R. The results agreed with other methods. Anthroyl DAG derivatives were applied by Thevenon et al. [47] for the measurement of phospholipid molecular species in biological samples (Fig. 6). The sensitivity allowed subpicomole measurements. They also reported the measurement was

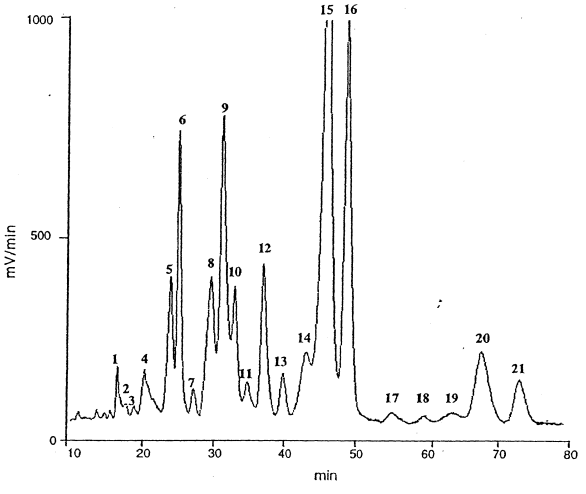


Fig. 6. Molecular species separated from diacylglycerophosphocholine (PC). Reproduced with permission from Ref. [47].

applicable to other bioactive lipids with one hydroxyl group, like hydroxyeicosatetraenoates (HETEs).

Other phospholipids besides glycerophospholipids can also be derivatized after hydrolysis. Mita et al. [48] hydrolyzed PAF and then derivatized it to form 7-methoxycoumarin ester. HPLC with FL detection was used to quantitate PAF derivatives with a lower detection limit at about 100 pg. Parlesak and Bode [49] analyzed lipopolysaccharide with derivatization after hydrolysis using HPLC. Reaction schemes are shown in Fig. 7. They measured lipid A based on the quantitation of β-hydroxymyristic acid and β-hydroxylauric acid. The β-hydroxy acids were methanolyzed and then derivatized with 9-anthracene-carboxyl chloride, 9-fluorene-carboxyl chloride and 4-(1-pyrenyl) butyric acid chloride and quantified by HPLC–FL, as shown in Fig. 8. The limit of detection was in the picogram level for real samples.

As discussed above, this strategy is very useful for phospholipid and its subclass analysis. For those phospholipids that have no free amino or hydroxyl group, it is better to hydrolyze them first and then derivatize with various reagents. Since glycerophospholipids are the main class of phospholipids, this method appears to be the most commonly used.

3.1.3. Miscellaneous phospholipids derivatizations

Besides the above two main approaches, there are still other derivatization methods for phospholipids with different structures. Most of these compounds have active groups to be derivatized directly, such as lyso-PAF, monophosphoryl lipid A and so forth. There are several approaches focused on characterization and quantitation of these minor phospholipids.

In body fluids, PAF is difficult to detect because it transforms to lyso-PAF rapidly, which has no biological activity [50], preventing the detection by bioassay. Lyso-PAF has a hydroxyl group instead of

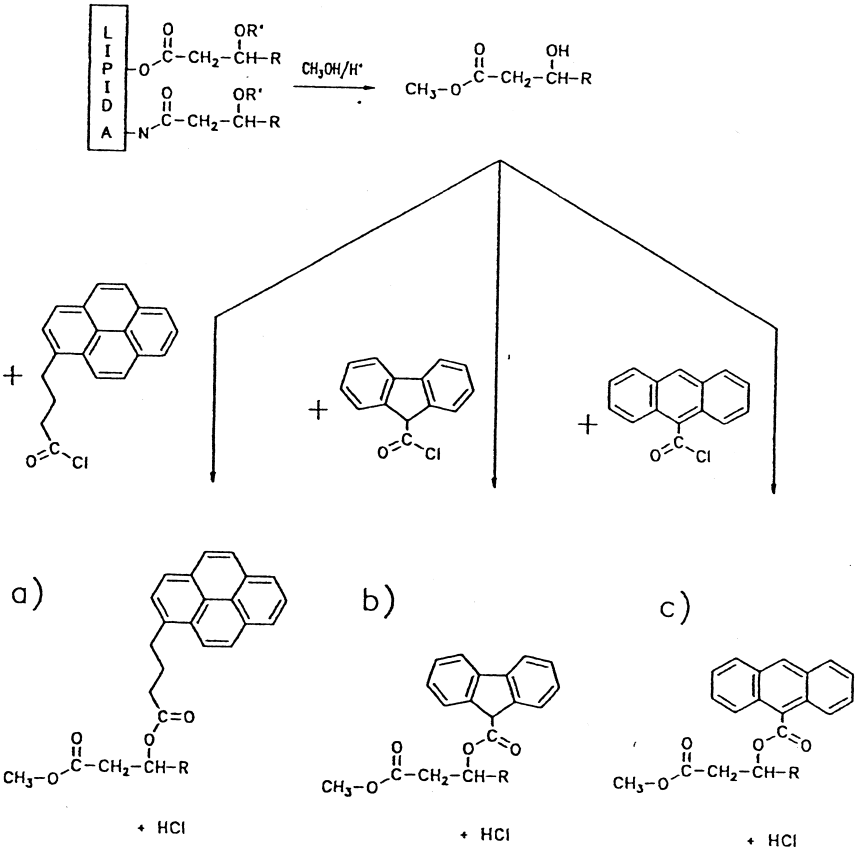


Fig. 7. Formation of β-hydroxy fatty acids from lipid A after methanolysis and derivatization with 9-anthracene-carboxyl chloride, 9-fluorene-carboxyl chloride and 4-(1-pyrenyl) butyric acid chloride. Reproduced with permission from Ref. [49].

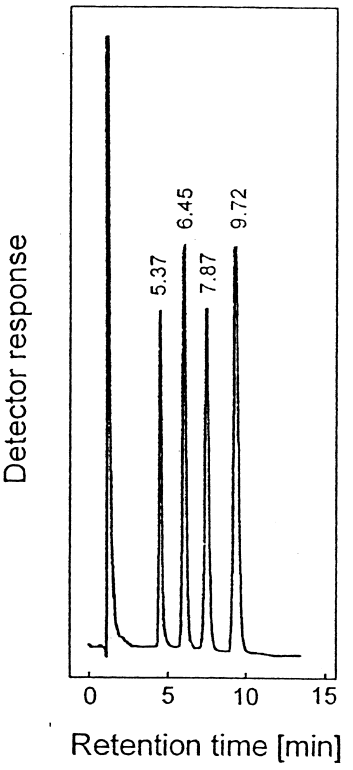


Fig. 8. Chromatogram of four derivatized products from β-hydroxy fatty acids. Reproduced with permission from Ref. [49].

an acyl group at the 2-position of the glycerol backbone, which is suitable for derivatization. Salari and Eigendorf [51] acylated the hydroxyl group in lyso-PAF with six different fluorescing fatty acids, and 9-anthracenepropionic acid was found to be the most efficient with a molar ratio (9-anthracenepropionic acid/lyso-PAF) over 2:1. This approach was quite sensitive (≥1 ng) and used to detect lyso-PAF in rabbit blood. Also, this technique was equally effective for other lyso-phospholipids, such as lyso-PI, lyso-PC and lyso-PE.

Recently, Hagen et al. [52] reported a HPLC method to analyze monophosphoryl lipid A (MLA) by derivatizing dinitrobenzyloxyamine (DNBA) to its 1-hydroxyl group. The DNBA-MLA was separated by RP-HPLC using tetrabutylammonium dihydrogenphosphate as an ion-pairing reagent to block phosphate groups and determined by UV at 254 nm.

Some derivatizations of phospholipids were achieved using post-column fluorescent derivatization [53–55]. Postle [53] used an FL probe, 1,6-diphenyl-1,3,5-hexatriene (DPH) to detect molecular species of PC, and later Kitsos et al. [54] used the same probe to monitor dipalmitoylphosphatidylcholine (DPPC) levels in rabbit eustachian tube washings, under the influence of ambroxol. Ouhazza and Siouffi [55] used another post-column derivatization reagent, 2,5-bis-[5-*tert*.-butyl-2′-benzoxazoly]-thiophene (BBOT), to detect PC, PG and sphingomyeline by TLC and HPLC. BBOT was first reported by Kraus et al. [56] to be able to complex with phospholipids. BBOT reacted with phospholipids specifically, so that it can be used for complex samples.

3.2. Derivatizations in GC–MS

In GC, analyte derivatization changes the characteristics of phospholipids by decreasing polarity and increasing the sensitivity of detection. Phospholipids are alkylated, esterified or acylated to a less polar form and separated by GC. To become GC compatible, phospholipids have been usually hydrolyzed by phospholipase C and derivatized with *tert*.-butyldimethylsilyl (TBDMS) [57,58], trimethylsilyl (TMS) [59,60] and pentaflurobenzoyl (PFB) [61]. A review of phospholipid analysis after enzymatic hydrolysis by GC was given in 1985 [62]. This technique provided a rapid analysis, however, hydrolysis of glycerophospholipids often yields two isomers: 1,2-diglycerides and 1,3-diglycerides, complicating the analysis. Hydrolysis of sphingomyelin also has artifacts due to ceramide degradation. A one-step quantitation of phospholipids was highly desired.

Satsangi et al. [63] and Weintraub et al. [64,65] developed methods to derivatize phospholipids directly with pentafluorobenzoyl chloride (PFB) and heptafluorobutyric anhydride (HFB). This technique is especially useful in characterization of PAF. Derivatization procedures are shown in Fig. 9. The reaction mixture was dried under nitrogen and then partitioned between hexane and water. The glycerol portion was in the hexane layer, while the polar head group was recovered from the water layer and treated with TMS. Therefore, a full structure elucidation was achieved. Comparably, phospholipase C treatment of phospholipids makes the detection of the polar head group difficult, due to the purification problem. Later

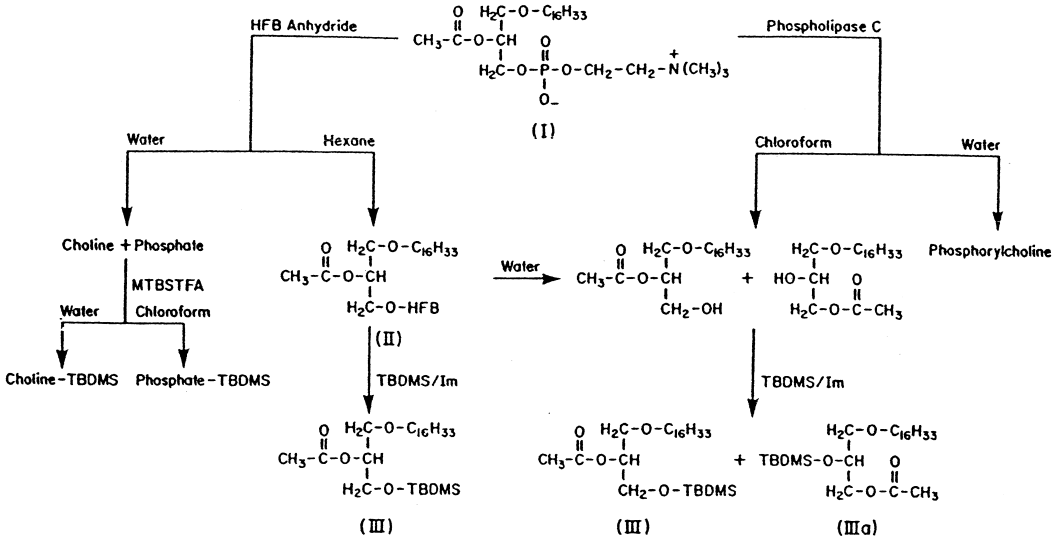


Fig. 9. Derivatization procedures for analysis of PAF. Reproduced with permission from Ref. [63].

on, Woodard et al. [66] quantitated PFB-derivatized PAF in normal human saliva. Various species of PFB-derivatized PAF in saliva were separated first by GC and quantitated by mass spectral analysis using standard calibration curves. Recently, this derivatization technique was used to analyze PAF after isolation by solid-phase extraction (SPE) [65].

Balazy et al. [67] quantitate PAF and other alkylether phospholipids at picogram levels by GC–MS via direct derivatization similar to the technique mentioned above. Van Kuijk et al. [68] reported a GC–MS method for the determination of phospholipid peroxides. PFB esters were formed with the hydroxyl group of phospholipids and used for determination of phospholipid peroxides. The detection level was as low as 10 pg by negative ion chemical ionization GC–MS.

4. Conclusions

As phospholipids are such an important class of compounds, the identification and quantitation of these in biological samples becomes a significant research and development area. Certain methods with high efficiency and high sensitivity are highly desirable. With HPLC and UV or FL detection, chemical derivatization has greatly improved the detection limits of phospholipids. Various reagents and approaches have been tried to identify and quantitate phospholipids so far. In these derivatizations, some are done directly, while others are achieved indirectly, after hydrolysis. With modifications, GC has also been applicable in phospholipid analysis. In summary, chemical derivatizations provide rapid separations with low detection limits. It will continue to play an important role in phospholipid analysis whether in HPLC, CE, GC or other methods.

5. Nomenclature

BBOT	2,5-bis-[5-*tert.*-butyl-2′-benzoxazoly]-thiophene
CD	cyclodextrin
CE	capillary electrophoresis
DAG	diacylglycerol
DNBA	dinitrobenzyloxyamine
DNS	1-dimethylamino-naphthalene-5-sulfonyl
DPH	1,6-diphenyl-1,3,5-hexatriene
DPPC	dipalmitoylphosphatidylcholine
FAB	fast atom bombardment
FL	fluorescence
FQ	3-(2-fluroyl)quinoline-2-carboxaldehyde
GC	gas chromatography

HETE	hydroxyeicosatetraenoate
HFB	heptafluorobutyric anhydride
HPCE	high-performance capillary electrophoresis
HPLC	high-performance liquid chromatography
IPD	indirect photometric detection
LIF	laser-induced fluorescence
LOD	limit of detection
LPS	lipopolysaccharide
LSD	light-scattering mass detector
MALDI	matrix assisted laser desorption ionization
MECC	micellar electrokinetic capillary chromatography
MLA	monophosphoryl lipid A
MS	mass spectrometry
NBD-Cl (F)	7-chloro(fluoro)-4-nitrobenzo-2-oxa-1,3-diazole
OPA	*ortho*-phthaldialdehyde
PAF	platelet activating factor
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PFB	pentafluorobenzoyl
PI	phosphatidylinositol
PITC	phenylisothiocyanate
PS	phosphatidylserine
SPP	sphingosine-1-phosphate
TBDMS	*tert*.-butyldimethylsilyl
TLC	thin layer chromatography
TMS	trimethylsilyl
TNS	6-*p*-toluidineo-2-naphthalenesulfonic acid
UV	ultraviolet

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