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Review

High-performance liquid chromatography of phosphatidic acid

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Abstract

This paper reviews existing high-performance liquid chromatographic (HPLC) methods for the analysis of phosphatidic acid (PA) in various sample matrices. In addition to the introductory background discussion on important aspects of PA in lipid biochemistry, the review provides comprehensive coverage in the areas of derivatization techniques, detection methods, and HPLC separation techniques. Conversions of PA to suitable derivatives enhance the detection sensitivity and improve the chromatographic behavior of the analytes. Detection methods include the use of state-of-the-art detectors and are discussed in terms of sensitivity, specificity, and compatibility with analytical systems. Pertinent normal-phase and reversed-phase HPLC data for PA are compiled from published methods.

Contents

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List of abbreviations

AcN	Acetonitrile
ADP	3-(9-Anthryl)diazo-2-propene
BTTA	1-Benzyl-3-p-tolytriazene
t-BuOMe	*tert.*-Butyl methyl ether
DAN	5-Dimethylamino-naphthalene-sulfonyl
DAP	di-[3-(9-Anthryl)-2-propenyl]
DGAC	Diglyceride acetates
DPG	Diphosphatidylglycerol
DTAP	Dodecyltriethyl ammonium phosphate
ELS	Evaporative light scattering
FI	Flame ionization
FL	Fluorescence
GPL	Glycerophospholipid
ISP	Isopropanol
LPC	Lyso-PC
LPE	Lyso-PE
MS	Mass spectrometry
ODS	Octadecylsilica
PA	Phosphatidic acid
PADM	PA dimethyl ester
PAMM	PA monomethyl ester
PAMP	PA monopropyl ester
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PS	Phosphatidylserine
PTAP	Pentyltriethyl ammonium phosphate
QAAP	Quaternary alkyl ammonium phosphate
RI	Refractive index
TBAP	Tetrabutyl ammonium phosphate
THF	Tetrahydrofuran
TMAP	Tetramethyl ammonium phosphate
TPS	2,4,6-Triisopropylbenzene sulfonyl chloride

1. Introduction

Phosphatidic acid (PA), 1,2-diacyl-*sn*-glycerol-3-phosphate, is the basic structural moiety for all glycerophospholipids (GPLs) (Fig. 1). As an important metabolic intermediate, the negatively charged PA coexists in very low abundance with various other GPLs in cellular membranes of

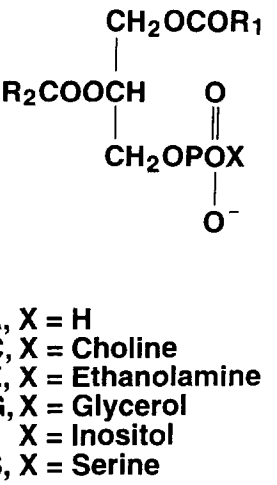

Fig. 1. General structure of PA and related GPLs. R_1 and R_2 represent respective alkyl and alkenyl groups of the fatty acid chains.

animals and plants. In lipid biosynthesis, PA is formed in both animal and plant tissues from 3-*sn*-glycerophosphate by enzymatic esterification of the phosphate with a fatty acyl-CoA or acyl-ACP in the presence of an acyl transferase [1–5]. Upon its formation, PA plays a pivotal role in a series of biotransformations for conversion to triacylglycerols, glycosylglycerides, and GPLs [6–8]. The latter polar lipids are known to be involved in lipoprotein and prostaglandin biosyntheses including the blood clotting mechanism [9]. Among many other activities of physiological significance, PA demonstrates specific biological activities serving as an endogenous calcium ionophore and a monnasyl acceptor in physiological processes [10–12]. Recently, PA has been shown to have a crucial involvement in signal transduction and cellular proliferation [13–16].

Since the first detection of PA in cabbage leaves [17], it has been shown that enzymatic action through phospholipase D on glycerophosphatides is responsible for the production of the acidic polar lipid in some vegetables [18]. The amount of PA found in soybeans varies markedly as the phospholipase activities in the plant seeds change. Consequently, quantitation of PA in soybean seeds is not only dependent on procedural variations in analytical methods, but

also on the condition of storage and methods for the processing of oilseeds [19–22]. Degummed soybean oil contains predominantly PA [23,24].

Lecithins, closely related to PA, are very useful commercial GPL products from soybean oil comprising phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) as the major GPLs, and lyso-PC (LPC) and PA as the minor GPLs. They find wide applications in food industries as emulsifiers, wetting agents, and antioxidants, and have gained importance in biomedical research as dietetic agents and therapeutants. Having its molecular formula as the key structural element shared by all GPLs, PA is an integral part in the GPL biosyntheses. The commercial and biomedical utility of PA is considered as valuable as commercial lecithin and other GPL products because PA has been shown to be an indispensable precursor to other classes of the polar lipids. In view of the multifaceted benefits offered by the polar lipid of physiological significance, analysis of PA can provide useful information for specific metabolic and neurological studies and for other studies in many biomedical research areas involving membrane biochemistry. Assessment of polar lipid profiles is important because chemical and physical properties of cell membranes can be affected by alteration of GPL compositions.

Chromatographic methods have been commonly used for the isolation, separation, and quantitation of PA along with other lipids in tissue samples. Of the various techniques, thin layer chromatography (TLC) was the most widely used method in the past for the analytical scale separation and qualitative/quantitative analyses of GPLs, in spite of the laborious and time-consuming procedures for quantitation. With the advent of modern separation technology, high-performance liquid chromatography (HPLC) has become the viable alternative and has proven to be the technique of choice for the analysis of PA with enhanced component resolution and analytical reproducibility.

A survey of the literature on HPLC of lipids reveals a considerable body of published work devoted to the use of this technique for the analysis of diverse mixtures of GPLs in various samples derived from animals or plants. Publications on the HPLC methodology for the analysis of PA are relatively sparse in contrast to the vast volume of comprehensive documentations available for the more abundant glycerophosphatides (e.g. PC, PE, and PI). The majority of reports on PA analysis have appeared in the literature as parts of research conducted in conjunction with the HPLC investigations of other GPL classes. In a direct analysis of PA with instrumentation of moderate sensitivity, due to the trace amounts of PA in natural sample matrices coupled with its highly polar ionic property, it often escapes detection. As result, analysis of PA has been difficult and requires a high degree of sophistication in methodological skills for the improvement of detection sensitivity and analyte separability in given chromatographic systems. In light of the lack of literature information concerning specifically the HPLC methodology of PA, this paper intends to provide a thorough coverage of topics dealing with the HPLC methods for the analysis of PA, the vitally important endogenous substance.

2. Derivatization techniques

Inspection of the PA structure (Fig. 1) indicates that the ionic property and the acidic functionality are too polar and interactive to be amiable with regular HPLC column systems. Chemical derivatization normally reduces the molecular polarity and thereby warrants a reproducible and sensitive analysis with a better defined PA analyte for the detection and chromatographic separation.

2.1. Phosphatidic acid ester derivatives

The easiest way (Fig. 2, scheme 1) to prepare a simple derivative of PA is by conversion to its dimethyl ester, PADM [25,26]. A practical and very useful derivatization procedure is described as follows: An ethereal solution of PA (pK_a ca. 3.5) in acid form (the salt form is first acidified with HCl to pH 2.5) is mixed with excess 0.1 *M*

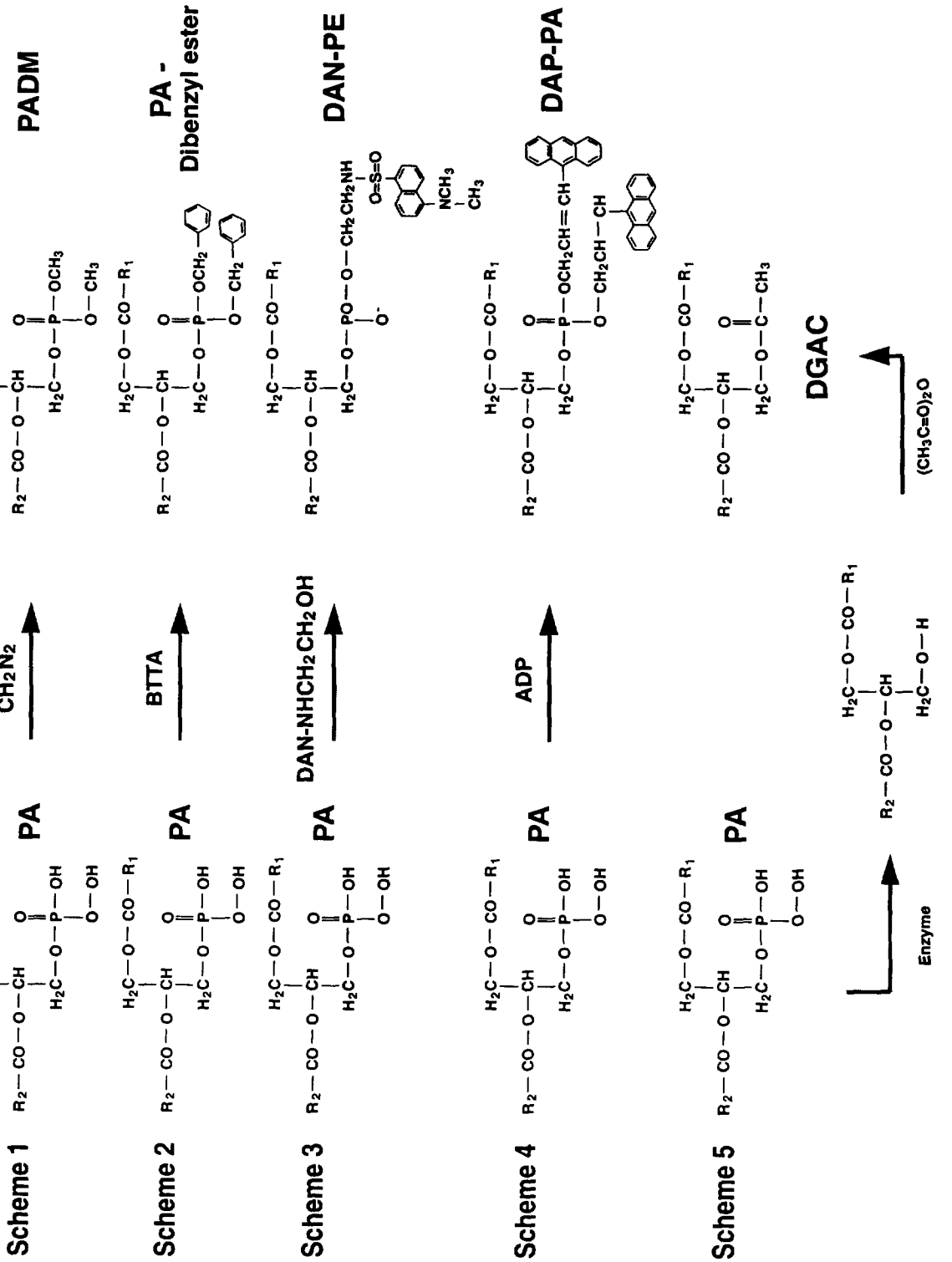

Fig. 2. Conversion of PA to its derivatives.

diazomethane for overnight at room temperature. After removal of solvent under nitrogen, the crude product is purified by TLC using a solvent of carbon tetrachloride–chloroform–glacial acetic acid–ethanol (120:80:1:6, v/v) for the development of chromatographic bands or spots [27]. The dimethyl ester on a TLC plate can be detected by using a reference sample of PADM stained with iodine. Direct treatment of several types of glycerophosphatides with diazomethane also produces PADM [26,28,29]. The monomethyl ester of PA, PAMM, is the intermediary product of the methylation reaction with diazomethane. It is rapidly converted to PADM in the presence of excessive amounts of diazomethane. However, methanolysis of PA or certain GPLs [PC, PE, phosphatidylserine (PS), or phosphatidylglycerol (PG)] with phospholipase D yields exclusively PAMM [26,30,31]. The same enzymatic reaction with the phosphatidyl acceptors in the primary alcohol series of lower-carbon-chain alkyls gives the monoalkyl esters of PA in quantitative yields [31,32].

For trace analyses, it is rather beneficial to introduce UV chromophores to PA molecules for enhancing analyte detectability. Benzylation of PA with 1-benzyl-3-*p*-tolytriazene (BTTA) [33] affords its dibenzyl ester in high yield (Fig. 2, scheme 2) [34]. Alternatively, when a high degree of detection sensitivity is desired for the PA analysis, the acidic moiety in the head group can be tagged with fluorophores to form fluorescent derivatives. Thus, reaction of PA with a mixture of 5-dimethylamino-naphthalene-sulfonyl (DAN)-ethanolamine (2 molar equiv.), 2,4,6-triisopropylbenzene sulfonyl chloride (TPS) (5 molar equiv.), triethylamine, and pyridine proceeds smoothly to furnish the dansylated product, Dan-PE, in which a single negative charge remains (Fig. 2, scheme 3) [35,36]. On the other hand, fluorescence labeled-PA can be prepared, via a novel approach, directly from a reaction of PA with 3-(9-anthryl)diazo-2-propene (ADP) [37] to give a highly fluorescent material, di-[3-(9-anthryl)-2-propenyl]-PA (DAP-PA) (Fig. 2, scheme 4) as the product [38,39].

2.2. Diglyceride derivatives

A number of derivatization methods that traditionally involve partial molecular destruction of phosphatides to remove the head group, in which the phosphoroglycerol bond is selectively hydrolyzed by phospholipase C, have been favored for analytical procedures [40]. Subsequent esterification of the resulting diradylglycerols with chromogenic or fluorigenic reagents produced the diglyceride esters suitable for the chromatographic analysis of the polar lipids. Dephosphorylation of PA occurs by hydrolysis with specific PA-phosphatases isolated from liver mitochondria/microsomes [41,42] or from wheat germ [43]. The phosphatidate phosphohydrolase of lung cytosol has also been used for the conversion of PA to diacylglycerol [8]. Subsequent to the dephosphorylation step, the mixture of the liberated diacylglycerides can then be acetylated with acetic anhydride and pyridine at 37°C for 3 h to give the diglyceride acetates (DGAC) (Fig. 2, scheme 5) [44,45]. It should be noted that acyl migration may occur during the acetylation process [46]. Other potentially useful arylcarbonyl derivatives of diglycerides include benzoates [47], *p*-nitro- [48], and dinitro-benzoates [49,50], and *p*-anisoates [51,52]. In addition, derivatization of a DMF solution of diacylglycerides derived from PA with α-naphthylisocyanate (200-fold molar excess) and 1,4-diazabicyclo(2,2,2)octane (4-fold molar excess) at 85°C for 2 h yields the naphthylurethane product, which can be sensitively analyzed by fluorescence detection in the picomole range [53]. Similarly, the 7-methoxycoumarin [54] and naproxen [55] derivatives of diradylglycerides exhibit excellent fluorescent properties for use in studies by HPLC–fluorescence detection.

3. Detection methods

There are several detection techniques applicable for the detection of PA analytes. The selection of a detector for a particular analytical project is contingent on the level of detection

sensitivity required, the purpose of the analysis, and the nature of the lipid analytes. Accordingly, the detectors used in HPLC systems for the PA analysis encompass a great deal of detection devices to monitor the column effluents: UV spectrophotometers, fluorescence (FL), refractive index (RI), evaporative light scattering (ELS) and flame-ionization detectors (FI), mass spectrometers (MS), radioactivity detectors, and phosphorimeters. Of these, the UV detection approach has been the most commonly adopted technique in lipid research.

3.1. UV spectrophotometric detection method

By virtue of its relative convenience, simplicity, and the low cost of UV detectors, detection of PA analytes in the UV-range (200–210 nm) has been a very pragmatic technique in PA analyses. However, severe analytical limitations exist due to the low molar extinction coefficients of the underivatized lipid molecules especially where the fatty acids contain few or no unsaturated bonds. The disaturated species of PA can virtually escape UV detection. Among the many solvents available for HPLC separation work, there is only a narrow spectrum of solvents that are transparent within the limits of UV wavelengths for lipid analyses. Qualitative analyses using a UV detection method serve restricted purposes for isolation and separation studies [56–58]. Variations in UV absorption with the fatty acid compositions, that in turn are a function of tissue origins and metabolic states of PA, can incapacitate the direct estimation of analytes by UV detector responses. Therefore, quantitative analyses of PA can only be achieved with UV detection by calibration with reference standards [59–61] or determination of PA phosphorus contents in isolated samples [60–63]. In a favorable situation, conversion of PA into a UV absorbing benzyl or arylcarbonyl derivative enables direct quantitation of the analytes of interest by integration of UV detector signals monitored at a longer wavelength of the UV chromophore beyond the 200–210 nm range [34,47–52].

3.2. Fluorescence detection method

Since fluorescence yields of lipids labeled with fluorophores are susceptible to changes in HPLC mobile-phase solvent composition, fluorescence detector responses in HPLC under gradient elution by no means reflect compositional distribution of analyte components in samples [64,65]. Hence, the fluorimetric detection methods employed in conjunction with gradient HPLC analyses suffer from definitive analytical drawbacks despite attainment of the high sensitivity and specificity of the fluorescence techniques. Nevertheless, quantitative analysis of [1-myristoyl-2-{12-(1-pyrenyl)dodecanoyl}]-PA with fluorescence detection in the gradient mode can be achieved through the normal channel of calibration with standards [66]. From a different perspective of HPLC elution techniques, direct isocratic HPLC quantitation of a number of fluorescence labeled PAs with fluorescence detection have been reported [35,36,38,39,53].

3.3. Differential refractometric detection method

Because refractive indices fluctuate considerably with changes in gradient mobile-phase conditions and other HPLC parameters, differential refractometric detection methods for lipid analyses are not feasible with the use of gradient elution. They are generally limited to isocratic HPLC assays or preparative separations, and find few applications in quantitative analyses of polar lipids [67] partly due to the lack of detection sensitivity. In one instance, the detector has been used in the qualitative evaluation of PADM derived from egg PC [68,69].

3.4. Evaporative light scattering detection method

A new universal, versatile evaporative light scattering detection system has recently been introduced which has proven to be remarkably successful in its application to the analysis of various non-polar neutral lipids and polar lipids. Interfaced with HPLC instruments, the ELS

detection has been used for quantitative analyses of PA along with other glycerolipids under gradient elution [24,70,71]. In HPLC investigations, usually good, reliable quantitative analysis can be obtained with the ELS detectors, provided the volatilities of the mobile phase systems meet the detector requirements and instrument parameters are optimized [72]. In a normal operation, the HPLC eluent may contain only small amounts of ammonium salts, as the detector is not compatible with most other types of salts and buffers. This is the primary obstacle to using an ELS detector in attempts to separate the molecular species of underivatized PA by ion-pair HPLC [61,73]. In view of the restrictions of inorganic buffer usage in mobile phases, relatively little use has been made of ELS detectors for the reversed-phase HPLC analysis of intact PA molecular species.

3.5. Flame-ionization detection method

Very similar to ELS detectors, flame-ionization detectors are versatile, and have high application potential for the quantitative analysis of lipids. They perform well only with relatively volatile solvents devoid of inorganic salts. A few HPLC investigations on naturally abundant glycerophosphatides using transport FI detectors have been described [74,75]. To a rather limited extent, utilization of this detection technique to the analysis of PA admixed with other GPLs has been studied by a group of investigators [76–78]. The FI detectors are not suitable for the detection of intact PA molecular species resolved with HPLC mobile phases in which inorganic ions are present.

3.6. Mass spectrometric detection method

Mass spectrometers are expensive and sophisticated instruments. Beside the universal and sensitive detection capability, the MS detection methodology can provide useful analytical data and valuable structural information about unknown lipid compounds. Analyses of MS spectra obtained by direct-probe fast-atom bombardment MS of egg PA (derived from egg PC) and dipalmitoyl-PA allow the characterization of respective fatty acid compositions [79] and the fatty acid fragmentation pattern [80]. However, the progress in on-line HPLC–MS of PA has been sluggish because of technical difficulties associated with low levels of PA in samples from natural origins, with the suitability of HPLC mobile phases used in the separation of underivatized PA species [61,73], and with the interface devices for sample introduction and ionization. Complex mixtures of PA molecular species have not been studied by LC–MS. The LC–thermospray-MS techniques employed in the qualitative and semiquantitative assessment of molecular species of the more abundant polar lipids [81–83] may be applied to study the species of PA derivatives. A most recent report on quantitative analyses of GPLs by LC–electrospray-MS showed that a molecular species 18:0–22:6-PA was identified along with those of PC, PE, PI, and PS [84]. The LC–electrospray-MS technique can provide greater detection sensitivity than the thermospray method [82,83].

3.7. Radioactivity detection method

In recent years, with the development of radioactivity detectors, the use of radiolabeled (^{14}C, ^{3}H, and ^{32}P) lipids has become increasingly important for the study of lipid metabolism. Measurements of radioactivity in lipid analyses can generally offer unusually high detection sensitivity. Individual molecular species of PA in animal tissues have been converted to [^{14}C]PADM upon treatment with [^{14}C]diazomethane and the radioactivity of the PA dimethyl ester quantitated by liquid scintillation counting [85]. Further, diacetate derivatives of [^{3}H]PA isolated from [2-^{3}H]glycerol-prelabeled cells can be separated by reversed-phase HPLC–UV followed by collection of the molecular species fractions and by off-line quantitation with a radioactivity detector [86]. The commercial availability of on-line HPLC-radioactivity detection units reduces analysis times and im-

mensely facilitates lipid metabolism research. In addition, the use of mobile phases containing inorganic salts should pose no complications at the interface of the HPLC–radioactivity detection system.

3.8. Phosphorimetric detection method

An automated phosphorus analyzer coupled with an HPLC system has been used for the quantitative analysis of lipid mixtures containing PA [87]. This detection method complements the UV-method for the direct quantitation of molecular species of underivatized PA in various samples when synthetic standards are not available for calibration [61].

4. HPLC separation techniques

4.1. Normal-phase HPLC

Analysis of PA in the normal-phase HPLC mode provides qualitative and quantitative data for PA as a lipid class. Separations of the PA class from other classes of non-polar neutral lipids and polar lipids depend heavily not only on the type and complexity of the lipid mixtures, but also on many chromatographic variables such as mobile phases, stationary phases, and elution modes. From a mechanistic standpoint, normal-phase separations of individual lipid classes are believed to proceed predominantly via an adsorption mechanism. Therefore, the differential polarity of the lipid components is the major factor contributing to the success in the separation of compounds of interest. Class analyses of PA in various lipid mixtures are more often carried out under gradient conditions than under isocratic elution in order to bring individual lipid classes of different polarity within the range of reasonable retention times. As gathered from earlier discussions, the choice of the mobile phase solvent systems depends heavily on the availability of suitable detectors.

Table 1 summarizes published information on normal-phase HPLC of underivatized PA (UV, FI, or ELS detection) along with two of its fluorescent derivatives (FL detection). Examination of the HPLC experimental variables compiled in Table 1 reveals that all but seven of the normal-phase HPLC methods employed gradient elution techniques. In essence, a gradient elution method is advantageous for the separation of multi-component lipid samples to accommodate the wide range of lipid polarities. Investigators have used different modes of gradient methods that include flow-rate gradients, composition gradients, and solvent gradients. As to the solvent selection, three general types of mobile-phase solvent systems have been used: (1) chloroform–methanol–water/ammonia (9 methods), (2) hexane–isopropanol–water (15 methods), and (3) acetonitrile–methanol–water (3 methods). Of these, more than one half of the mobile phases contain acids, bases or electrolyte buffers to alleviate chromatographic problems associated with peak broadening and peak tailing of PA as well as other acidic charged GPLs. It is noteworthy that a gradient HPLC experiment with a type 1 mobile phase (chloroform–methanol–water/ammonia) tends to yield high-efficiency resolution of GPL components accompanied by sharper chromatographic peaks (methods 22–25, Table 1) (Fig. 3A,C). Examples of normal-phase HPLC separations obtained under various conditions are shown in Fig. 3A–E.

With the exceptions of methods 20 and 21 where fluorescent derivatives of PA were assayed with a fluorescence detector, most workers (13 methods) have utilized a UV detector for the analysis of the underivatized PA (Table 1). The recent increase in the application of an ELS detector in PA analysis (7 methods) provides impetus for future expansion of HPLC research in this area. The prospect of the HPLC–ELSD methodology for the PA analysis would be encouraging if the interface technology should develop new ELS detection systems to improve solvent compatibility and detection sensitivity.

Under the conditions employed in the methods listed in Table 1, the retention times of PA in sample mixtures ranged from 3 to 35 min, except for those from methods 1 and 2 in which PA eluted at 95 and 125 min, respectively. Cursory perusal of normal-phase HPLC profiles in pub-

Table 1
Normal-phase HPLC methods for the separation of underivatized PA

(Method No.) Column	Mobile phase solvent (elution mode); elution order	Retention time (min) (detector)	References
(1) Corasil II 37–55 μm	Chloroform→ methanol–8% ammonia (gradient); PG > PE > PI > PC > PA > LPC	95 (FI)	76, 77
(2) Corasil II 37–55 μm	Pentane→ ether–chloroform→ methanol–8% ammonia (gradient); PG > PE > PI > PC > PA > LPC	125 (FI)	77, 78
(3) Corasil II	Chloroform–methanol–ammonia (50:35.9:7) (isocratic); PE > PS > PI > PC > PA > LPC	15.3 (FI)	88
(4) Lichrosorb Si-60 10 μm	Hexane–isopropanol–water (6:8:1.3) (isocratic); PA > PE > PG > PC	4.3 (UV)	58
(5) Lichrosorb Si-60 10 μm	Hexane–isopropanol–water (6:8:0.75) →(6:8:1.4) (gradient); PA > PE > LPE > PI > PS > PC > LPC	6.1 (UV)	58
(6) Spherisorb S10W	Hexane–isopropanol–water (6:8:0.75) →(6:8:1.75) (gradient); PA > PI > PE > PS > PC > LPC	4.7 (UV)	56
(7) μBondapak-NH_2 10 μm	Chloroform–[methanol–water (25:1)] (20:8) (isocratic); PA > PG > PS > PC > PE	3 (FI)	99
(8) MicroPak Si-5 5 μm	Hexane–isopropanol–water–H_2SO_4 (97:3:0:0.02) → (75:24:0.9:0.1) (gradient); PA > DPG > PG > PS > PI > PE > LPE > PC > LPC	6.4 (UV)	57
(9) LiChrosorb Si-60 10 μm	Hexane–isopropanol–acetate buffer (8:8:1) 0.3→2.3 ml/min (gradient); PE > PC > PI > PA	9.6 (UV)	89
(10) LiChrosorb Si-60 10 μm	Hexane–isopropanol–0.2 *M* AcOH (8:8:1) 0.5→3 ml/min (gradient); PE > PA > PI > PC	5.8 (UV)	89
(11) LiChrosorb Si-60 10 μm	Hexane–isopropanol–water (8:8:1) 1→5 ml/min (gradient); PE > PI > PA > PC	13.7 (UV)	89
(12) μPorasil	Acetonitrile–methanol–water (80:15:6.5)→ (50:45:6.5) (gradient); PA > PE > LPE > PC > LPC	24 (^{31}P)	87
(13) LiChrospherSi-100 10 μm	Hexane–isopropanol–25 m*M* phosphate–ethanol–acetic acid (367:490:62:100:0.6) (isocratic); PE > PA > PI > PS > PC > LPC	16.8 (UV)	62
(14) MicroPak Si-10 10 μm	Acetonitrile–methanol–85% H_3PO_4 (130:5:1.5) (isocratic); PI > PS > PE > LPE > PA,PG > PC > LPS > LPC	10.4 (UV)	90
(15) LiChrosorb Si-100 5 μm	Hexane–isopropanol–water (60:80:7)→(60:80:14) (gradient); PE > PI > PS > PA > PC	32 (UV)	91
(16) μPorasil	Hexane–isopropanol–water (6:8:0.5) →(6:8:1.5) (gradient); PG > DPG > PE > PI > LPE > PA,PS > PC > LPC	25.9 (UV)	59

(*Continued on p. 290*)

Table 1. *Continued*

(Method No.) Column	Mobile phase solvent (elution mode); elution order	Retention time (min) (detector)	References
(17) Bio-Sil HP 10 10 μm	Acetonitrile → acetonitrile–water (80:20) (gradient); PA > PG > PI > PE > PS > PC > LPC	7.8 (UV)	92
(18) LiChrosorb Si-100 10 μm	Chloroform → chloroform–methanol–ammonia (gradient); DPG > PA > PG > PE > PI > PC > PS > LPC	13.7 (ELS)	70
(19) Spherisorb 3 μm	Hexane–isopropanol–water (57.8:39:3.2) → (52.6:42:5.4) (gradient); PE > PG > PI > PA > PC	16.6 (ELS)	93
(20) Nucleosil 5 μm	Ethylacetate–hexane–methanol–(Et_3NH (70:30:7.5:0.01) (isocratic); PA	20 (FL)	39
(21) RadialPak Si 10 μm	Isooctane–tetrahydrofuran → isopropanol–CH_2Cl_2 → isopropanol–water (gradient); PG > PE > PA > PS > PC > LPC	26 (FL) (pyrene-PA)	66
(22) LiChrospher-Si-60 5 μm	Chloroform–methanol–ammonia → chloroform–methanol–water–ammonia (gradient); PE > PI > PS > PC > PA > LPC	13 (ELS)	71
(23) LiChrosorb Si-60 10 μm	Chloroform–tetrahydrofuran → chloroform–methanol–ammonia (gradient); PE > PI > PS > PA > PC	26,35 (ELS)	24, 73
(24) LiChrosorb Si-100 5 μm	Chloroform–tetrahydrofuran → chloroform–methanol–$N(Et)_3$ (gradient); PE > PI > PS > PA > PC	27 (ELS)	73
(25) LiChrospher Si-60 II 3 μm	Chloroform–*tert.*-butyl methyl ether → chloroform–methanol–ammonia (gradient); PE > PI > PA > PC	31 (ELS)	60
(26) Cyclobond-I 5 μm	Hexane–isopropanol–ethanol–water/TMAP (isocratic); PE > PC > PI > PA	23 (UV)	60
(27) LiChrosorb Si-60 5 μm	Hexane–isopropanol–isopropanol/water (40:46:14) → (40:4:56) (gradient); PE > PI > PA > PC > LPC	33.4 (ELS)	94

Abbreviations: DPG = diphosphatidylglycerol; LPE = lyso-PE; THF = tetrahydrofuran; t-BuOMe = *tert.*-butyl methyl ether; TMAP = tetramethyl ammonium phosphate.

lished methods (Table 1) indicated that adequate separations of PA from other lipid classes were observed in most cases (21 out of 27 methods). HPLC experiments with methods 9–11, 14, and 16, however, led to unresolved or partially resolved PA from other GPL class components. Depending on the kind of mobile phase additives and the mode of flow-rate gradients, clusters of PA/PI and PA/PI/PE were partially resolved in methods 9 and 10, respectively, while a broad band attributable to PA appeared to separate from other lipid components as reported in method 11. With method 14, in spite of the good separation of a host of polar lipid classes with a mobile phase of acetonitrile–methanol–85% H_3PO_4 under isocratic elution, PA coeluted with PG and incompletely separated from PC. Under compositional gradient elution with a hexane–

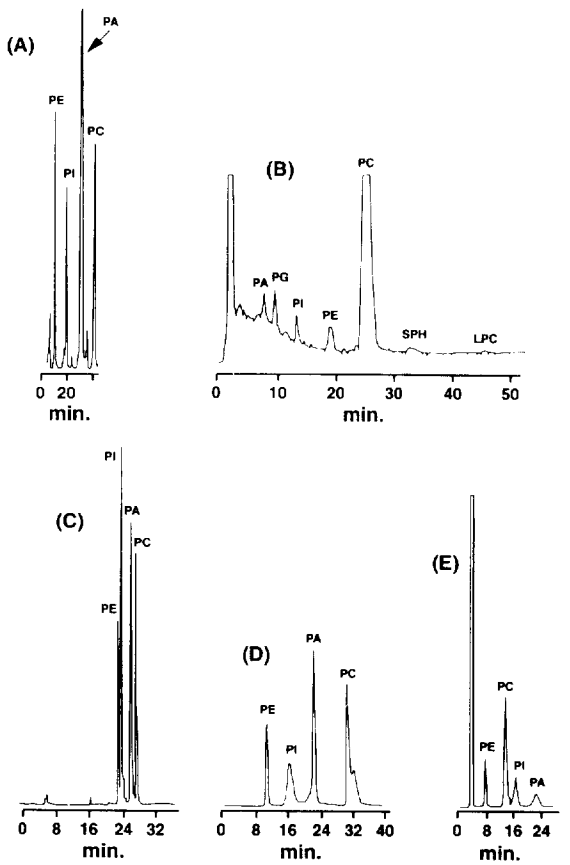

Fig. 3. (A) Normal-phase gradient HPLC–ELS detection of GPLs isolated from soybeans stored at high moisture (14%). Column, Lichrosorb Si-60, 10 μm; solvent A = chloroform–tetrahydrofuran (1:1, v/v), solvent B = chloroform–methanol–ammonia (1:92:7, v/v); gradient, A → B → B → A (30 → 15 → 10 min). Flow-rate, 1 ml/min. (redrawn from Ref. [24] with permission). (B) Normal-phase gradient HPLC–UV detection of polar lipids in adult human lung lavage. Column, Bio-Sil HP 10, 10 μm; solvent A = acetonitrile–water (80:20, v/v), solvent B = acetonitrile; gradient, A (12.5%) → A (12.5%) → B (25%) → B (25%) (5 → 10 → 35 min). Flow-rate, 2 ml/min. (redrawn from Ref. [92] with permission). (C) Normal-phase gradient HPLC–ELS detection of GPL standards on γ-cyclodextrinsilica. Solvent A = chloroform, solvent B = chloroform–methanol–water/15% Et_3N (1:92:7, v/v); gradient, A → B → B → A (30 → 10 → 10 min). Flow-rate, 0.5 ml/min. (D) Normal-phase isocratic HPLC–ELS detection of GPL standards on γ-cyclodextrinsilica. Mobile phase, chloroform–methanol–water/15% Et_3N (50:15.5:32:2.5, v/v). Flow-rate, 1 ml/min. (E) Normal-phase isocratic HPLC–UV detection of GPL standards on β-cyclodextrinsilica. Mobile phase, hexane–isopropanol–ethanol–water/5 m*M* TMAP (pH 6.3) (35:32.7:26.8:5.5, v/v). Flow-rate, 1 ml/min. (from Ref. [60] with permission).

isopropanol–water mobile phase as described in method 16, the PA peak overlapped with that of PS.

As illustrated in Table 1, the elution sequence of PA in samples containing other GPL classes varies quite dramatically with the combined effects of mobile phases and stationary phases used in the methods. In all but one method (method 18) using a type 1 mobile phase (chloroform–methanol–water/ammonia) system, the acidic PA eluted later from a column as expected based on its stronger adsorption on the stationary phase in the basic mobile-phase medium. Conversely, HPLC with type 2 mobile phases (hexane–isopropanol–water) yielded elution patterns in which PA emerged from a column with little retention, although exceptions were found in methods 15, 16, 19, 26, and 27. Parallel to the observation described, PA seemed to show relatively weak adsorption in comparison to other lipid components in normal-phase HPLC with type 3 mobile phases (acetonitrile–methanol–water). An exception to this generalization existed in method 14 where an isocratic technique was adopted with the type 3 mobile phase containing 85% phosphoric acid.

It should be pointed out that in published normal-phase HPLC methods (Table 1), lipid mixtures of various sample matrices of animal and plant origins were more frequently used. For stationary phases, most workers chose the conventional silica columns that were available commercially. In addition, two silica-based phases bonded with polar alkylamino- (method 7) and cyclodextrin- (method 26) groups have been used for the normal-phase separation of mixtures of PA and other GPLs. Both methods 7 and 26 employed isocratic elution techniques. The apparent reversed elution order of PA in the sample mixtures analyzed by the two methods might partly result from the use of different mobile phases.

4.2. Reversed-phase HPLC

Owing to the existence of various fatty acid chains in the non-polar tail groups of PA molecules (Fig. 1), reversed-phase HPLC techniques

Table 2
Reversed-phase HPLC methods for the separation of PA

(Method No.) Column	Mobile phase solvent (elution mode); sample source	Derivative	References
(1) μBondapak C_{18}	Acetonitrile–water (95:5) (isocratic); egg PC	Dibenzyl ester (UV)	34
(2) Partisil 10 ODS 10 μm	Methanol–water (96:4) (isocratic); egg PC	Dimethyl ester (RI)	69
(3) Partisil 10 ODS 10 μm	Acetonitrile–water (95:5) (isocratic); hydrogenated egg PC	Dimethyl ester (FI)	69
(4) Partisil 10 ODS in tandem	Acetonitrile (isocratic); egg PC, soybean PC	Dimethyl ester (RI)	95
(5) LiChrosorb RP 18 5 μm	Methanol–water (86:14) → (96:4) (gradient); rat lung microsome PA	Diacyglycerol naphthylurethane (FL)	8, 53
(6) LiChrosorb RP-18 5 μm	Acetonitrile–isopropanol–*tert.*-butyl methyl ether–water (83:10:5:2) (isocratic); human T-cell PA	Diacylglycerol acetate (UV, ^3H)	86
(7) Econosphere C_{18}	Methanol–10 m*M* KH_2PO_4 (95:5) (isocratic); egg PC	Dansyl ethanolamine ester (FL)	36
(8) LiChrosorb RP-18 5 μm	Acetonitrile–isopropanol–methanol–water (50:27:18:5) (isocratic); rat liver PC, pig leukocyte PC, rate liver microsome PA	Dimethyl ester (UV)	63 96, 98
(9) Chromagenetics ODS-2 in tandem	Methanol–water (88:12) (isocratic); synthetic Lyso-PA	Dimethyl ester lyso-PA (^{32}P)	97
(10) NovaPak C_{18} 4 μm	Acetonitrile–methanol–water /5 m*M* PTAP (70:22:8) (isocratic); egg PC	PAMM (UV)	73
(11) Spheri-5 RP-8 5 μm	Acetonitrile–methanol–water /1.5 m*M* DTAP (65.8:13.7:20.5) (isocratic); egg PC	PAMM (UV)	73
(12) Adsorbsphere HS C_{18} 5 μm	Acetonitrile–methanol–water /1.25 m*M* TBAP (70:22:8) (isocratic); egg PC	PAMM (UV)	73
(13) YMC-ODS 5 μm	Acetonitrile–methanol–water /50 m*M* TMAP (49:49:2) (isocratic); egg/wheat/brain/soybean PC	PA (UV)	61
(14) Ultrasphere ODS 5 μm	Acetonitrile–methanol–water /20 m*M* TBAP (70:26:4) (isocratic); egg PC	PA (UV)	61

Abbreviations: PTAP = pentyltriethyl ammonium phosphate; DTAP = dodecyltriethyl ammonium phosphate; TBAP = tetrabutyl ammonium phosphate. For others, see footnote to Table 1.

are ideally suited for the separation of PA molecular species. However, the underivatized PA complex that consists of different molecular species shows little tendency to be resolved in reversed-phase HPLC systems without modifications of the PA structure or mobile-phase conditions. Under the unmodified circumstance, solvophobic interactions between a hydrocarbonaceous stationary phase and the polar head group of the parent PA solutes are apparently weak in chromatographic separation processes. Therefore, chemical derivatization techniques are essential assay procedures for the analysis of PA molecular species.

Table 2 compiles 14 literature methods for the separation and quantitation of derivatized (12 methods) and underivatized PA (2 methods) molecular species. The PA species have been analyzed as their dibenzyl-, dimethyl-, monomethyl-, or dansyl ethanolamine-esters (methods 1–4, 7–12). Also, their diacylglycerol-naphthylurethane (method 5) and acetate (method 6) derivatives have been used in studies of PA molecular species. For the selection of mobile phases, investigators have employed three general types of mobile phases: (1) acetonitrile–water (3 methods), (2) methanol–water (4 methods), and (3) acetonitrile–methanol–water (7 methods). From the experience with all three types of mobile phases, authors have chosen type 3 mobile phases for our reversed-phase HPLC work on PA molecular species (methods 10–14). Taken into consideration baseline stability, isocratic methods have been used in reversed-phase HPLC of PA species having somewhat similar hydrophobicity. There is a sole example (method 5) in Table 2 in which the HPLC analysis was carried out under gradient elution. In other words, unless the analyte species contain a high degree of disparity in fatty acid chain lengths, investigators have preferred isocratic to gradient elution procedures in the analysis of PA molecular species (Table 2).

Careful review of the HPLC parameters for the methods given in Table 2 indicates that earlier methods 1–4 for the analysis of dimethyl and dibenzyl esters of PA produced chromatograms giving no baseline separations and poor peak symmetry. In these methods, modifications of the PA head groups via chemical derivatization alone appear to be technically insufficient to eliminate PA adsorption on μ-Bondapak C_{18} or Partisil 10-ODS columns used in methods 1–4. This phenomenon is presumably due to interactions of the analyte solutes with residual silanol of alkyl-bonded silica causing the observed peak tailing. Such silanol effect might well be minimal in methods 5 and 6 where PA molecular species were ascertained as diacylglycerol derivatives without the polar phosphoryl head groups. Removal of peak tailing can also be effected by incorporation of a dansyl ethanolamine group into the PA molecule and by addition of an electrolyte buffer to the mobile phase used (method 7).

With recent advances in column technology, separations of molecular species can be done with relatively small particle size (3–5 μm) column packings to increase resolution efficiency. The commercial availability of stationary phases with improved surface coverage and endcapping features has expedited the development of analytical methods for the separation of ionic compounds and hard-to-resolve mixtures. In reversed-phase ion-pair HPLC methodology, mobile phases for the ionic analytes call for variable concentrations of counter-ion electrolytes with controlled pH values. Recent HPLC studies (methods 10, 12–14, Table 2) have shown that excellent baseline separations were attained in reversed-phase HPLC of the negatively-charged PAMM or PA on octadecylsilica (ODS) using ion-pairing techniques. In these HPLC analyses, cationic quaternary alkyl ammonium phosphates (QAAP) were added to the type 3 mobile phase (acetonitrile–methanol–water). Since columns of 4- or 5-μm ODS packings were used in these later methods, separations of PA molecular species were more efficient than those published earlier using methods 1–4. Furthermore, a modification of the conventional simple ternary solvents void of any salt by adding electrolytes to the mobile phase afforded symmetric chromatographic peaks with minimum tailing, notwithstanding the prevalence of the highly polar ionic PA analyte solutes.

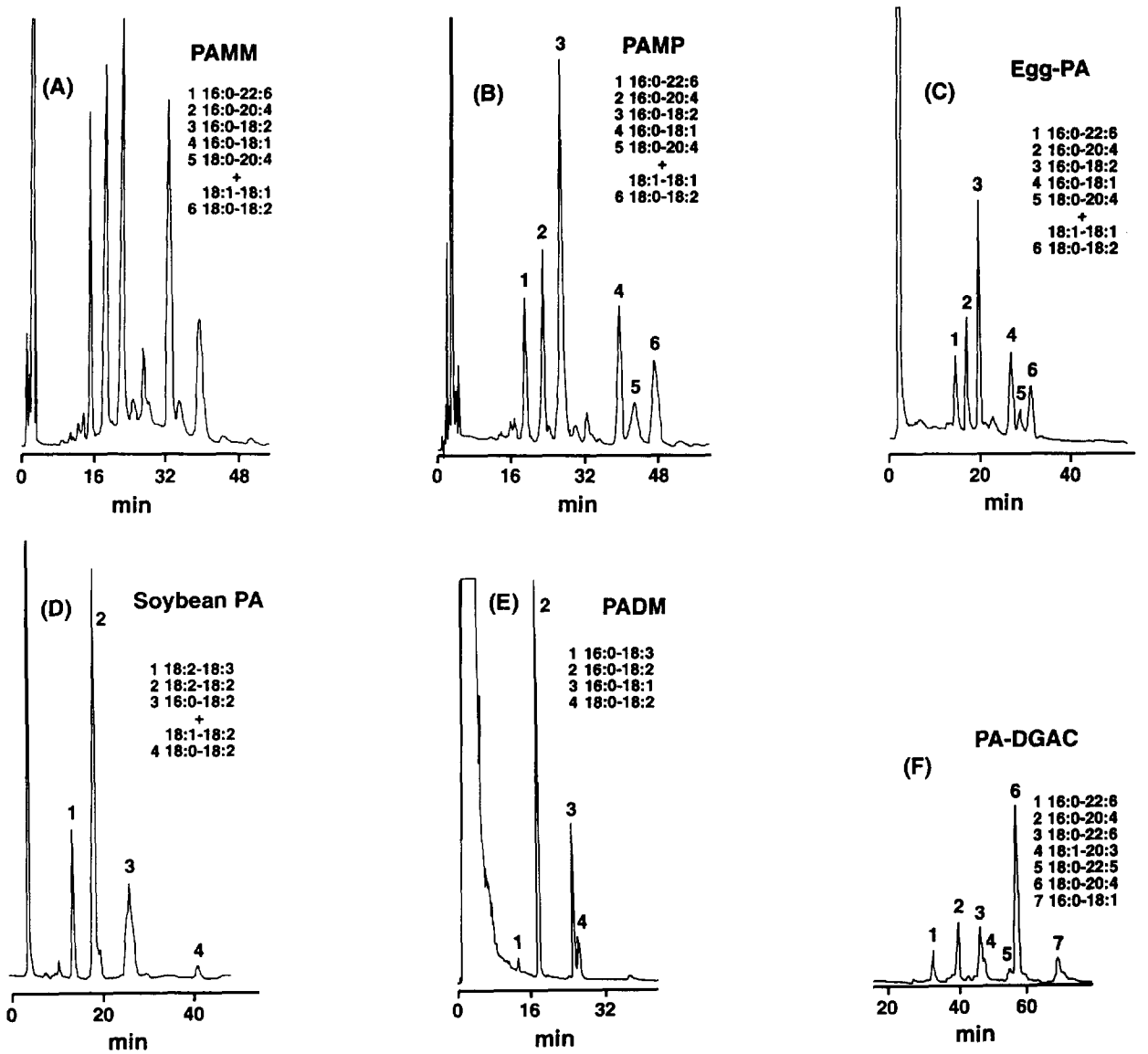

Fig. 4. (A) Reversed-phase isocratic HPLC–UV detection of egg PA monomethyl ester (PAMM) on a NovaPak C_{18} column. Mobile phase, acetonitrile–methanol–water (70:22:8, v/v) containing 0.5 m*M* DTAP at pH 6.5. Flow-rate, 1 ml/min. (B) Reversed-phase isocratic HPLC–UV detection of egg PA monopropyl ester (PAMP). Conditions same as in Fig. 4A, except that the mobile phase contained 5 m*M* PTAP. (C) Reversed-phase isocratic HPLC–UV detection of egg PA on a Ultrasphere C_{18} column. Mobile phase, acetonitrile–methanol–water (49:49:2, v/v) containing 50 m*M* TMAP at pH 6.5. Flow-rate, 1 ml/min (from Ref. [61] with permission). (D) Reversed-phase isocratic HPLC–UV detection of soybean PA. Conditions: same as in Fig. 4C. (from Ref. [61] with permission). (E) Reversed-phase isocratic HPLC–ELS detection of PA dimethyl ester (PADM) in a genetically engineered soybean oil on a NovaPak C_{18} column. Mobile phase, acetonitrile–isopropanol–methanol–water (50:27:18:5, v/v). Flow-rate, 1 ml/min. (F) Reversed-phase HPLC–UV detection of PA-[^{3}H]diacylglycerol acetate (PA-DGAC) in stimulated JURKAT cells, a human T-cell leukemia line. Column, LiChrosorb RP-18. Mobile phase, acetonitrile–isopropanol–*tert.*-butyl methyl ether–water (83:10:5:2, v/v). Flow-rate, 0.5 ml/min (redrawn from Ref. [86] with permission).

As compared to reversed-phase ion-pair HPLC of PA monomethyl ester, PAMM, the possession of two negative charges on the underivatized PA renders it mandatory to increase the ionic strength of QAAP counter-ions to achieve a comparable degree of component resolution as those for PAMM (methods 13 and 14 vs. methods 10–12, Table 2). Fig. 4A–C present chromatograms showing reversed-phase HPLC separations of molecular species of egg PAMM, egg PA monopropyl ester (PAMP), and the underivatized egg PA (all derived from egg PC), respectively. In comparison with the egg PA samples, fewer components were observed in the HPLC trace of a soybean PA sample (derived from soybean PC) (Fig. 4D). An important aspect of ion-pairing techniques used in methods 10–14 stems from the plausible manipulation of the QAAP concentration and the size of its alkyl groups to acquire the degree of separations desired (Fig. 4A vs. 4B). Linear correlation between capacity factors (k') and the total number of QAAP provides a unique means to predict the type of unknown QAAP or unknown k' values of analyte species. The principal drawback of the ion-pair reversed-phase HPLC techniques (methods 10–14) for PA analyses is the limitation inherent with UV detection as discussed before.

For economical reasons, few lipid manufacturers produce authentic PA materials because fairly low levels of PA are present in natural products. Thus, all commercial PA compounds are literally derived from PC of the same natural origins. Table 2 shows various sample sources used in the reversed-phase HPLC methods for PA analyses. Numerous reversed-phase HPLC studies have been conducted to separate or quantify molecular species of commercial egg PA and/or soybean PA derived from corresponding PC (methods 1, 2, 4, 7, 9–14). Molecular species distributions of PA derived from hydrogenated egg PC have been assessed by method 3. Three groups of investigators have analyzed PA molecular species in animal tissues: (1) rat liver microsomes (method 5), (2) human T-cells (method 6), and (3) rat liver microsome, and PA derived from rat liver PC and pig leukocyte PC. In the authors' laboratory, molecular species of PA found in genetically modified soybean oil samples have been determined as PADM derivatives (Fig. 4E). With reference to the various known methods (Table 2) for reversed-phase HPLC of PA, analysts have monitored column effluents with UV- (8 methods), FI- (2 methods), RI- (2 methods), FL- (1 method), and radioactivity detection (2 methods) techniques. The last detection technique has been utilized in concurrence with UV detection in the analysis of PA as its diglyceryl acetate in stimulated human T-cells (method 6) (Fig. 4F). Surprisingly, none of the published work has used ELS detection methods for the analysis of PA molecular species. In this regard, authors have routinely used an ELS detector for the analysis of PADM species in modified soybean oil (Fig. 4E).

4.3. Other HPLC techniques

Molecular species of PA dimethyl esters derived from egg PC have been separated on a silver-loaded Partisil-10-SCX column by HPLC under isocratic elution with diethyl ether [68]. Under the conditions employed, the PADM species having a higher number of double bonds eluted later from the column in accordance with the normal-phase elution sequence. In another recent HPLC study, the dimethyl esters of the PA complex derived from pig polymorphonuclear leukocyte PC were separated into alkylacyl and diacyl analogues on Lichrosorb-Si-60 [96]. The sample was eluted isocratically with hexane–isopropanol (95:5, v/v) in the normal-phase mode. To obtain sufficient quantities of the acidic polar lipid for subsequent studies, the PA class in soybean oil or lecithin samples has been separated and isolated by preparative HPLC [24,100].

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