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Review

Molecular species analysis of phospholipids

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Abstract

The elucidation of phospholipid molecular species composition provides detailed structural information concerning various lipids and thus offers descriptions of crucial determinants of membrane physical and biological properties. Various methods differing in labor intensity, mode of separation and detection, type of calibration, as well as other factors, have been published. Thus precision and accuracy are expected to vary considerably between methods. Qualitative and quantitative aspects of different procedures for molecular species analysis of individual phospholipid classes are discussed in this review. Special emphasis has been given to the characterization of biological tissue samples.

Keywords: Reviews; Phospholipids

Contents

1. Introduction

Phospholipids are found in all biological membranes and they consist of a mixture of different classes, such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidic acid (PA) and phosphatidylserine (PS). The occurrence of higher concentrations of other lipid families tends to be more organ specific. Examples of these are cerebrosides (brain), car-

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diolipin (heart muscle) and sphingomyelin (erythrocytes). Different tissues exhibit their own unique pattern of phospholipid classes and each class in turn exhibits a unique pattern of molecular species containing various combinations of fatty acids in different proportions. Positional distribution of fatty acids on the glycerophospholipid backbone almost unfailingly finds the least unsaturated fatty acid, generally saturates or monoenes, in the *sn*-1 position and the most unsaturated in *sn*-2. Accordingly, contemporary molecular species investigations may include this element of stereospecific analysis when confirmation is required for unexplored samples.

It is generally accepted that biomembrane composition may be altered due to nutritional, environmental or xenobiotic exposure. Fatty acyl distributions appear to be most easily affected [1], whereas lipid class composition is less amenable to alteration [2,3]. Changes in the total fatty acid compositional level must be related to a change in the molecular species composition of one or more of the structural lipid classes, at least in the absence of a large pool of free fatty acids, as for the case of the brain, for example. This has drawn attention to the importance of molecular species analysis of individual lipid classes. Even though some particular molecular species may subserve a more vital function than others, it is generally believed that there is a specific biochemical or structural function for each species as well as a reason for their abundance in a given tissue, as evidenced by numerous investigations [4,5]. In general, tissue phospholipids do not exhibit a random distribution of fatty acids that simply reflects the fatty acid composition of the plasma supply. The species composition has a genetic component as evidenced by organ and tissue specific molecular species patterns [6]. However, this aspect of composition may be influenced by stress such as that induced by starvation or alcohol abuse as well as nutritional challenge. These changes may be regarded as a means of cellular adaptation to environmental conditions in order to maintain vital functions. In other words, biological tissues seem to have a degree of freedom within which a fluctuation in molecular species composition is permitted. Beyond certain threshold values, the organ may be unable to function in an optimal manner. We may speculate that these boundaries become narrower for more vital peripheral organs. Brain and other nervous tissue lipids appear more resistant to change than those of other organs, especially in adulthood. The biochemical system responsible for the conservation of brain lipid composition may have emerged as an adaptive mechanism during evolution.

There is clearly a need for reliable analytical procedures for the characterization of phospholipid molecular species both in basic research and in industry. The food, cosmetic and pharmaceutical industries uses large amounts of phospholipid mixtures, such as lecithins [7] as emulsifying additives. Manipulation of the phospholipid molecular species composition in these lecithin preparations may provide a means of extending their range of functionality in many applications.

Published methods for phospholipid molecular species analysis generally include chromatographic separations in conjunction with pre- or post-column derivatization. Other methods may involve the analysis of intact molecules which require fewer preparatory steps. In this review, the advantages and disadvantages of analytical techniques employed for molecular species analysis will be discussed with a special emphasis on quantification of biological tissue samples.

2. Methods

2.1. Methods overview

Detailed elucidation of lipid composition has been the subject of research investigations for several decades [8,9]. In 1958, Inouye and Noda reacted egg phospholipids with mercuric acetate before submitting them to paper chromatography [10]. Chromatographic resolution was a function of differences in mercuration depending on the degree of unsaturation of the phospholipid fatty acyl chains. In the sixties, Collins published a series of papers [11–13] in which a method for the countercurrent distribution of rat liver PC molecular species was described. Separation was incomplete but provided some information on species composition. Renkonen [14–16], utilized acetolysis [17] and enzymatic hydrolysis with phospholipase C [18] followed by acetylation of the diglyceride residues and fractionation into sub-

classes based on degree of unsaturation by silver nitrate thin-layer chromatography (TLC) [19,20]. The different subclasses were then submitted to gas chromatographic analysis (GC) of the fatty acids. This method provided useful information about the relative abundance of fatty acids in different subfractions of chicken egg yolk, bovine brain and human serum phospholipids. It also provided a conceptual framework for future method development in subsequent decades.

Another approach was taken by Arvidson [21,22], where intact, underivatized, PC and PE were separated according to degree of acyl chain unsaturation on silver-impregnated TLC plates, generally referred to as silver-ion chromatography or argentation chromatography. Silver ions interact reversibly with double bonds to form complexes. Chromatographic retention is a function of the number and configuration of the unsaturations. Acetylated diacylglycerol derivatives of bovine brain polyphosphoinositides were subjected to this method by Holub et al. [23]. The mono-, di-, tri-, tetra-, penta- and hexaene bands were recovered and subjected to fatty acid analysis by GC. In other investigations using silver ion TLC, PE from egg yolk was analyzed [24] and underivatized rat liver PI was resolved into 4 bands (monoenes+dienes, tri-, tetra- and polyens) [25]. Similarly, Salem et al. [26] purified PS from bovine brain on a DEAE column and separated this intact class by silver-ion TLC. Two major spots were detected indicating the presence of two groups of molecular species differing widely in degree of unsaturation. The most retarded spot was primarily composed of 18:0/22:6-PS and the least retarded was composed of 18:0/18:1-PS. Yeung et al. [27] converted bovine brain PS into trifluoroacetamide derivatives and submitted them to silver ion TLC. The identities of the PS molecular species reported in this paper was 18:0/18:1 and 18:0/20:1. This paper also contains PS species information of pig brain and erythrocytes, rat liver and rabbit muscle.

The most frequently used procedure for phospholipid molecular species analysis appear to have been enzymatic hydrolysis with phospholipase C rendering 1,2-diacyl-*sn*-glycerols followed by conversion to either GC or high-performance liquid chromatography (HPLC) amenable derivatives. In HPLC applications, conversion to UV absorbing derivatives has been used to a high degree. Another method described *tert.*-butyldimethylsilyl (*t*-BDMS) ethers [28,29] for mass spectrometric detection. Blank et al. [30] utilized the chromophoric benzoate derivatives of diacyl-, alkylacyl- and alk-1-enylacylglycerol residues derived from bovine brain PE after phospholipase C hydrolysis, in an HPLC separation with UV detection at 230 nm. Similar procedures were also used by Patton et al. [31,32]. In GC applications, the 1,2-diacyl-*sn*-glycerols may be converted to corresponding monoacetyldiacylglycerols [33,34], trimethylsilyl (TMS) ethers or *t*-BDMS ethers. A general drawback of this approach is that it precludes analysis for metabolic studies, for example, the incorporation of ^{32}P or [^{14}C]choline. Methods employing multi-step derivatizations may subject the lipids to the danger of rearrangement of the fatty acyl chains on the glycerol backbone. It is also more time-consuming to obtain the derivatives, compared to analyzing a total lipid extract directly without any derivatization. Submitting underivatized/unmodified, i.e. "intact", phospholipids to chromatographic separation is only a viable option when the mobile phase is a solvent, as in HPLC and TLC. In the case of HPLC, some general difficulties are associated with detection [35], which will be discussed further below.

With the advent of lipophilic derivatives of Sephadex [36,37], a new solid phase for liquid chromatography was available which was used with some success for molecular species separations [38–40]. This partition mode of separation was much further improved when solid phases for HPLC composed of alkylated (usually C_{18} chains) silica particles became available. This has become one of the most utilized separation methods for molecular species characterization in liquid chromatography. Even though reversed-phase TLC plates are commercially available, they have found little use in phospholipid molecular species separation. The utilization of TLC for this category of separations is mostly confined to separation according to degree of acyl chain unsaturation, i.e. silver-ion chromatography.

More recently, liquid chromatography–mass spectrometry (LC–MS) methods have provided highly selective analysis of phospholipid species (reviewed by Kim and Salem) [41] and have also been used in the analysis of intact molecular species of bovine

milk sphingomyelin (N-acylsphingosine-1-phosphocholine) [42].

Several references to molecular species analyses of various tissues are found in Table 1.

2.2. Liquid chromatography

Molecular species separation of phospholipids by reversed-phase chromatography is based not only on the analyte's overall degree of unsaturation, as is the case in silver-ion chromatography, but also on differences in chain length.

Alkylated Sephadex derivatives were the first solid-phase with reversed-phase characteristics that were used for the separation of intact PC molecular species [43,44]. In 1979, Porter et al. [45] were the first to partially separate intact molecular species of egg yolk PC on a commercially available reversed-phase (C_{18}) column by isocratic HPLC with refractive index detection. Also in 1979, Crawford and Wells published a method for the separation of soybean PC and oxidized species thereof [46]. Smith and Jungalwala [47] resolved intact phosphatidylcholines from egg yolk, bovine brain and porcine liver by HPLC with UV absorbance detection at 205 nm. At about the same time, Patton et al. [48] achieved excellent species separations of underivatized rat liver PC, PE, PI and PS using a similar method with UV absorbance detection at 205 nm (Fig. 1). Collection of the peaks followed by phosphorous determination was used for quantification. Common to methods using underivatized phospholipids is that they conserve experimental economy compared to procedures which includes hydrolysis and derivatization steps, but they suffer from detection problems if UV at short wavelengths, where the degree of unsaturation of the analyte has a notable impact on signal intensity. Calibration becomes a very tedious task since each species needs an individual calibration curve. A further complication is that authentic standards for many molecular species might not be commercially available. Accordingly, the accuracy of the quantitative results obtained may be limited. Refractive index detection may be an alternative in some situations when the sample composition is not very complex. Refractive index is less versatile than UV as a lipid detection technique as it precludes the use of solvent gradients as a means to optimize chromatographic performance.

In order to improve on quantitative precision, Cantafora and Masella [49] developed an HPLC method in which an internal standard (1,2-diarachidine) was added before the lipids were converted into corresponding glycerobenzoate derivatives. The PC molecular species of bile and liver in both the human and the rat (Table 2) were investigated. Using statistical experimental design and multivariate optimization methods [50], Kaufmann and Olsson [51] focused upon improving chromatographic performance in the HPLC analysis of bovine milk PE and PC molecular species of which 11 and 24 peaks were resolved, respectively.

Table 1
Applications of animal tissue phospholipid molecular species analysis[a]

Tissue	Origin	Reference
Bile	Human	[84]
Brain	Monkey	[85]
Brain	Human	[86,87]
Brain	Fish	[88]
Brain	Rat	[89–94]
Pineal gland	Fish	[95]
Egg yolk	Hen	[96]
Erythrocytes	Human	[89,97]
Hearts	Rat	[98]
Kidney	Pig	[99]
Leukocytes	Rat	[100]
Liver	Mouse	[101]
Liver	Rat	[102–105]
Liver	Fish	[106]
Muscle	Fish	[107,108]
Muscle	Sheep	[109]
Muscle	Bovine	[110]
Plasma	Fish	[111]
Retina	Bovine	[111–115]
Retina	Frog	[116,117]
Retina	Rat	[118]
Retina	Fish	[95,119]
Roe	Fish	[120]
Sciatic nerve	Rat	[121]
V79-R cells (cultured)	Chinese hamster	[122]

[a] Other applications are discussed in detail in the text.

2.3. HPLC detection

Christie and Hunter [52] used the evaporative light-scattering detection principle (ELSD) [35,53]

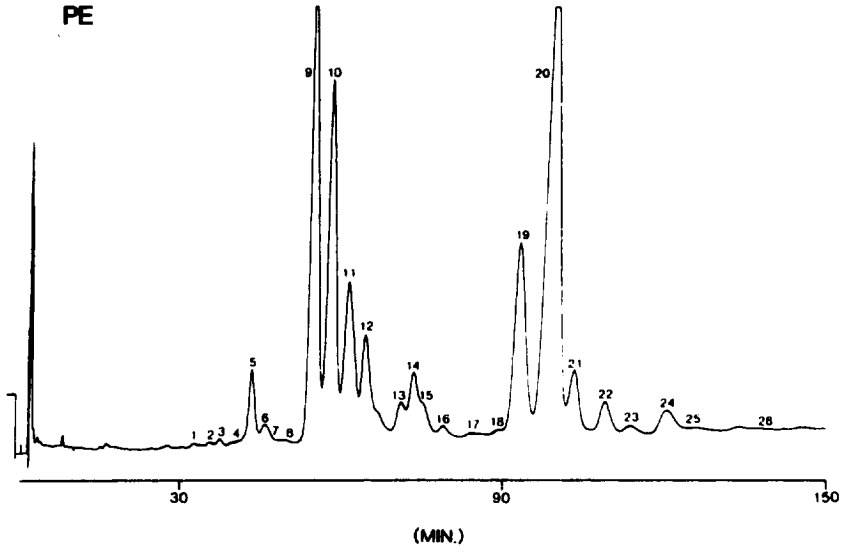

Fig. 1. HPLC separation of the underivatized molecular species of rat liver PE. Analytical column: Ultrasphere ODS (4.6×250 mm I.D.). Mobile phase: 20 m*M* choline chloride in methanol–water–acetonitrile 90.5:7:2.5 (v/v). Flow-rate: 2.0 ml/min. Detection: UV at 205 nm. Major peaks: 9=16:0/22:6, 10=16:0/20:4, 11=16:0/18:2, 12=18:1/18:2, 19=18:0/22:6, 20=18:20:4, 21=18:0/18:2. From Ref. [48], with permission.

for the reversed-phase separation of rat liver PC. Sotirhos et al. [54], compared the ELSD with UV detection in the analysis of intact of egg yolk PC and PE and also of rat liver PC. They found that the ELSD had several advantages over UV detection. The most important was that the response was largely independent of the number and configuration of double bonds in the fatty acid chains, thus making quantitation more straightforward. Since the ELSD was much less sensitive to baseline shift during gradient elution than the UV detector, greater freedom in the selection of solvents was permitted. This greatly expanded the systems available for improving chromatographic resolution.

The light-scattering detector has become increasingly used in the field of lipid analysis [35] but not many workers have utilized this detector for molecular species analysis of intact molecules. One reason for this might be that UV detectable lipid derivatives are perceived as somewhat more amenable to reversed-phase chromatography than intact phospholipids. Kaufmann and Olsson used this detector for the separation of molecular species of intact PC and PE from bovine milk [51]. They also employed the thermodynamic variable, column temperature, often overlooked in liquid chromatography, by making runs in the range between 75 and 95 °C. The light-scattering detector has been compared with the refractive index detector in terms of sensitivity and a systematic comparison has been made by Hopia and Ollilainen [55]. The sensitivity of the light scattering detector was 30 ng/injection compared to 50 ng/injection for the refractive index detector when injecting triolein and the relative standard deviation of responses was clearly better for the ELSD. Refractive index detection was used by Itoh et al. [56] in an isocratic separation of diacylglycerol acetates derived from rat lung PC. In this paper, a comparison is also made to UV detection at 205 nm; it is clearly shown that the di-16:0-PC species, which is barely detectable in the UV trace, is the principal species at 26.8% of total and that the main peak on the UV trace (16:0/20:4) only represents 7.5% of the material! This rather extreme example demonstrates that very large errors can be made with UV detector analysis.

Partial resolution of phospholipid molecular species was achieved by Van der Meeren et al. [57] on a normal-phase HPLC system with light-scattering detection. In this case, quantification was best

Table 2
Molecular species compositions of rat liver phosphatidylcholines

Reference	[48]	[73]	[49]	[28]	[29]	Reference	[48]	[73]	[49]	[28]	[29]
Molecular species[a]	%	%	%	%	%	Molecular species[a]	%	%	%	%	%
14:0/18:2w6	–	–	1.3	–	–	16:0/16:1w7	0.7	0.6	–	0.5	0.6
14:0/22:6w3	0.6	–	–	–	–	16:0/18:1w9	1.4[b]	5.2	8.3[b]	6.0[b]	6.7
16:0/18:2w6	14.4	15.2	19.9	17.3	14.6	16:0/18:1w7	–	1.7	–	–	1.4
16:0/20:3w6	1.5	–	–	–	–	17:0/18:1w9	1.1	–	–	–	–
16:0/20:3w6	1.3	[e]	1.1	–	–	18:0/18:1w9	3.4	1.6	2.6	1.0	1.2
16:0/20:4w6	9.7	16.6[e]	17.1	11.8	15.5	Total SFA/MUFA	6.6	9.1	10.9	7.5	9.9
16:0/20:5w3	2.1[d]	1.8[d]	–	2.6	0.8						
16:0/22:5w6	2.6[e]	–	–	1.2[b]	–	18:1w9/20:4w6	–	2.2	0.6	3.2	1.5
16:0/22:5w3	1.0	[f]	–	–	[d]	18:1w7/20:4w6	–	2.3	–	–	0.7
16:0/22:6w3	3.9	4.6	2.2	5.2	7.9[d]	Total MUFA/PUFA	–	4.6	0.6	3.2	2.2
18:0/18:2w6	14.8	12.8	14.3	13.6	13.2						
18:0/20:3w6	1.6	–	0.6	–	–	18:2w6/18:2w6	1.5	[d]	–	0.8	0.8
18:0/20:4w6	16.1	21.3	29.8	21.3	22.5	18:2w6/20:4w6	0.7	–	–	–	–
18:0/20:5w3	[e]	1.5	–	3.4	0.5	18:2w6/20:4w6	[d]	2.0[f]	–	0.9	0.6
18:0/20:5w6	1.4	1.5	–	–	–	18:2w6/22:6w3	1.2	–	–	–	–
18:0/22:5w3	3.3	–	–	0.6[b]	0.8	Total diPUFA	3.4	2.0	–	1.7	1.4
18:0/22:6w3	3.6	2.7	–	3.5	3.8						
20:0/18:2w6	–	–	1.1	–	–	16:0/16:0	–	0.9	–	0.9	0.5
Total SFA/PUFA[c]	77.9	78.0	87.4	80.5	79.6	Total diSFA	–	0.9	–	0.9	0.5
PLC hydrolysis	–	x	x	x	x	PLC hydrolysis	–	x	x	x	x
Benzoate deriv.	–	–	x	–	–	Benzoate deriv.	–	–	x	–	–
TMS-derivatization	–	x	–	–	x	TMS-dervatization	–	x	–	–	x
t-BDMS-deriv.	–	–	–	x	–	*t*-BDMS-deriv.	–	–	–	x	–
HPLC	x(205 nm)	–	x(230 nm)	–	–	HPLC	x(205 nm)	–	x(230 nm)	–	–
GC	–	x	–	–	x	GC	–	x	–	–	x
LC–MS	–	–	–	x	–	LC–MS	–	–	–	x	–

[a] Criteria for inclusion in table was if present above 0.5% in any of the data sets.
[b] No indication of double bond positions given.
[c] SFA=saturates, PUFA=polyunsaturates, MUFA=monoenes.
[d], [e] and [f]Co-migrating species within respective data set. The sum of co-migrating species given next to presumed major species.

when a calibration standard was employed that had a fatty acid composition that was similar to the samples.

The human erythrocyte plasma membrane transbilayer distribution of molecular species of PE was elucidated by Hullin et al. [58]. To this end, a covalent labeling technique was used that enabled the selective reaction of outer leaflet aminophospholipids, which were submitted to reversed-phase HPLC with diode-array detection after conversion to trinitrophenyl (TNP) derivatives. The polyunsaturated species were enriched in the membrane interior of PE and the monoene species dominated in the outer leaflet. It was later concluded [59] that dietary *n*-3 fatty acids were asymmetrically incorporated into human erythrocyte membranes after four weeks of fish oil administration.

Fluorescence detection was also used by Abidi et al. in the analysis of brain PS and PE as well as PE and PC from egg yolk [60]. Three different fluorescent labeled PE derivatives (dansyl, pyrenesulfonyl and fluorescein-thiocarbamoyl) were submitted to reversed-phase HPLC and the molecular species composition compared. The relative amounts of corresponding molecular species differed widely between the differently labeled phospholipids, illustrating the need for careful calibration. Separation was achieved by adding quaternary ammonium salts to the mobile phase and the fluorescein derivative was considered to provide the best resolution, but at

the expense of an 140 min analysis time. Intact molecular species were also separated by Postle [61] in conjunction with post-column derivatization and fluorescence detection. Prior to the formation of the fluorescence derivatives, a UV trace (205 nm) was recorded and used for retention time identification. The ratio of UV absorbance to fluorescence signal was calculated for each eluted peak and was a constant for each molecular species. The mean relative standard deviation of this procedure was estimated at 20%. This procedure has been used in metabolic studies in the guinea pig [62–65] and rat [66].

2.4. LC–MS

Kim and Salem developed a thermospray LC–MS method for the analysis of intact phospholipid species that suffered somewhat less from the detection problems discussed above [67,68]. It was suggested that the same calibration curve could be used for quantification of species [41] which possessed the same partition number (PN), i.e., the total number of acyl carbons less twice the total number of unsaturations [69]. The thermospray technique was utilized to study ethanol-induced alterations in molecular species of rat liver microsomal phosphatidylinositol (PI) [70]. Thermospray spectra provide fragment ions which carry detailed structural information for qualitative analysis of species composition, but response factors differ for species with differing amounts of unsaturation. The LC–MS technique thus provides an excellent solution to identification of molecular species in unknown materials. However, the positional distribution of fatty acids cannot be determined from the spectra. Ma and Kim [71] utilized reversed-phase chromatography and a mobile phase containing 0.5% (v/v) ammonium hydroxide for separation of intact phospholipid classes. This separation was used in conjunction with thermospray mass spectrometry for a qualitative description of molecular species composition of rat brain phospholipids. The detection limit was about 20 ng when using selective ion monitoring (SIM). Kim et al. have also employed electrospray LC–MS to further improve sensitivity and uniformity of detector response while maintaining very high selectivity (Fig. 2) [72]. The detection limit in this case was about 0.5 pmol, demonstrating improvement over the corresponding thermospray procedure.

The role of LC–MS in quality control laboratories is largely to establish the identity of peaks in HPLC chromatograms. HPLC, which generally is less disturbed by instrumental problems, is still the workhorse in quality control applications for non-volatile lipids. However, the future trend may lie in favor of the LC–MS combination. No other practical analytical technique is comparable in terms of sensitivity. New and more reliable ionization systems are now available, such as electrospray and discharge-assisted thermospray (plasmaspray) which promise to make LC–MS more comparable to HPLC in terms of reproducibility.

2.5. GC

Intact phospholipids are not suitable for GC analysis. They must first be converted into more volatile derivatives. This procedure may entail hydrolysis with phospholipase C, recovery of the 1,2-diacylglycerol phospholipid residues and conversion into trimethylsilyl (TMS) ethers, *t*-BDMS ethers or acetyl derivatives. Myher and Kuksis have used TMS [73] and *t*-BDMS [74] derivatives to study phospholipid molecular species compositions of many different biological tissues including rat liver PC [75]. Separation was achieved on a 15 m×0.32 mm I.D. RTx-2330 (Restec) fused-silica column, resolving 27 peaks, representing 33 different species. In another investigation, using this method, human plasma PC, PE and PI showed striking differences in molecular species composition [74]. The major peak in the GC profile of PI comprised 50 mole% and contained both 18:0/20:4*n*6 and 18:0/20:3*n*6. In PE, this peak was 22.7% and only 6.7% in PC. Analysis of human erythrocyte PC, PE, PI and PS revealed similar levels of the combined 18:0/20:4*n*6, 18:0/20:3*n*6 fraction (47.4% of PS) [76].

It is difficult to find comparable data on similar biological tissue samples in order to make qualitative and quantitative comparisons. However, in Table 2, molecular species data from five different papers using different analytical methods, including GC, have been compiled. The variation in the quantitative results listed in Table 2 exhibit a method-to-method

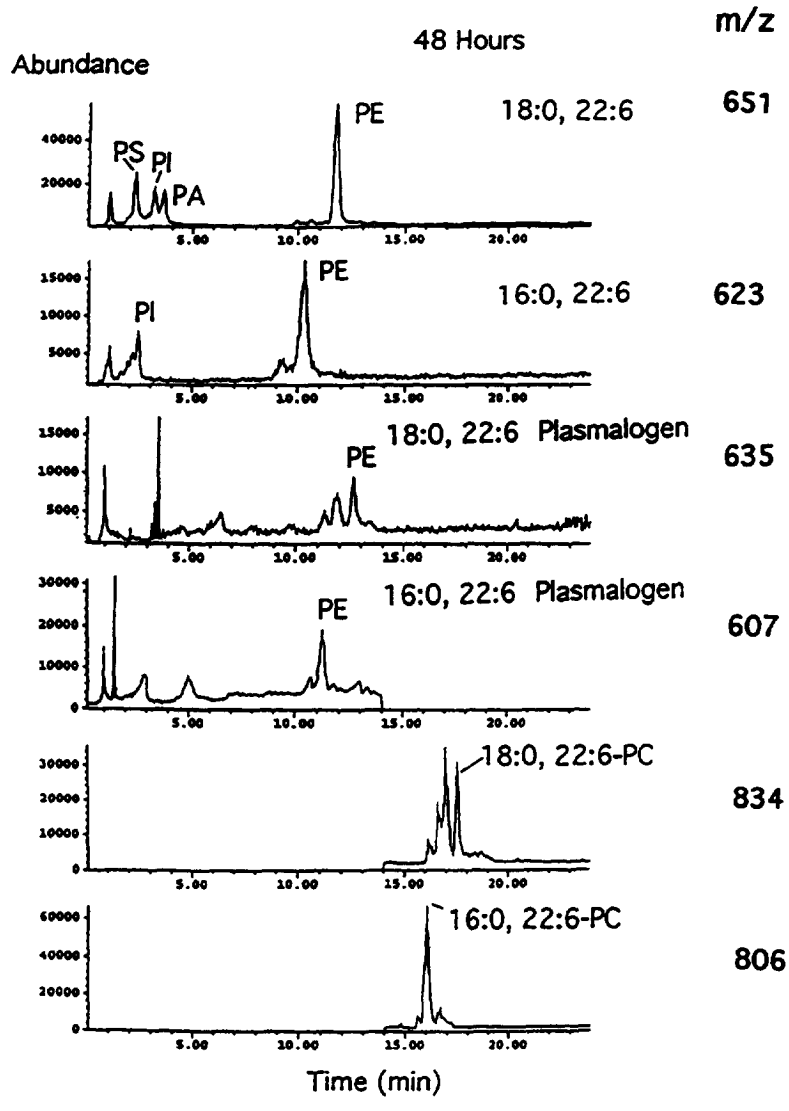

Fig. 2. Ion chromatograms of 22:6*n*3 containing phospholipid species obtained after incubation of C-6 glioma cells with 100 μM 22:6*n*3 for 48 h. Analytical column: C_{18} (2.1×150 mm I.D.). Mobile phase gradient: ammonium hydroxide (0.5%) in water–methanol–hexane, changing from 12:88:0 to 0:88:12 (v/v). Flow-rate: 0.5 ml/min. Detection: Mass spectrometry with electrospray ionization. From Ref. [71], with permission.

variation that is typical for methods using different detection systems and lacking inter-laboratory calibration. The peaks 16:0/20:4, 16:0/18:2, 18:0/20:4 and 18:0/18:2 are listed as the major species in all five papers while quantitation of the minor peaks appears quite variable.

2.6. Plasmalogen phospholipids

The analysis of mammalian tissues is often complicated by the presence of glycerol ether-containing lipids, such as alkenyl-acyl (plasmalogen) and alkyl-acyl analogues. These diradyl forms of individual

lipid classes may be isolated before the molecular species composition of each subclass is elucidated. Various diradyl analogues will typically elute within the same time-window on reversed-phase chromatography. If an LC–MS system is available, this might be less of a problem. The plasmalogens can comprise a large portion in some tissues and cannot always be neglected. Normal-phase liquid chromatography on unmodified silica adsorbents does not possess the power to resolve the plasmalogens within a defined phospholipid class due to insufficient structural differences, but Olsson et al. achieved separation between plasmalogen PE and diacyl PE from human brain cerebellum and grey matter [3], utilizing gradient HPLC with a DIOL column operated at 65 °C.

Curstedt [77] developed a method for the separation of alkenyl-acyl and alkyl-acyl PC from their diacyl analogues and their subsequent separation into individual molecular species. This involved cleavage of the PC molecules by phospholipase C, to remove the polar head-group, followed by separation of the diradyl-glycerols on a Lipidex-5000 liquid-chromatographic column or silver-ion TLC [14,78]. Chromatographic separation between these subclasses may also be achieved after dephosphorylation with phospholipase C followed by acetylation [79,80].

Free aldehydes can be obtained after hydrolysis of plasmalogens with concentrated hydrochloric acid followed by purification on preparative TLC [81]. Pugh et al. [82] submitted subgroups of bovine heart PC to mild alkaline (NaOH) and acidic (HCl) hydrolysis to render free aldehydes for further analysis. The liberated aldehydes can be reacted with fuchsin to form colored complexes which may be measured spectrophotometrically or converted into corresponding dimethylacetal derivatives and analyzed by gas chromatography [83].

3. Conclusions

The method chosen for molecular species elucidation depends in part on the complexity of the sample and the equipment available in the laboratory. Table 3 lists and compares the most commonly used methods. For qualitative purposes silver-ion TLC may be regarded as the most cost-efficient method for species separation. However, when quatitation is required, a scanning densitometer for in situ measurements also becomes necessary. Silver-ion HPLC is a better way to conduct separations since the silver is protected from exposure to light. Even though these HPLC columns appear quite stable, leakage of

Table 3
Molecular species analytical methods evaluation[a]

	Liquid chromatography						Gas chromatography	
	Silver-ion		Reversed phase				FID	MS
	TLC Densitometry	HPLC[b] UV/RI/ELSD	HPLC UV	RI	ELSD	MS		
Intact PLs	Yes	Yes	(Yes)[c]	(Yes)[d]	Yes	Yes	No	No
Resolution	2	3	4	2[d]	4	4	5	5
Sensitivity	1	[b]	3	1	1	5	4	5
Quantitation	3	[b]	3	3	4	4	5	5
Identification	3	[b]	3	3	3	5	4	5
Sample preparation	5	[b]	2	2	5	5	1	1
Investment[f]	5[e]	4	4	4	4	1	3	2

[a] Rating scale: 1–5, with 5 indicating highest or best.
[b] In most aspects the same as for reversed-phase HPLC.
[c] Detection of saturates greatly impaired.
[d] Gradient elution not possible which prevents optimal chromatographic resolution.
[e] Quantitative TLC with *in situ* densitometry approx. the same as HPLC.
[f] Highest equipment investment=1, lowest=5.

silver, which might have a negative impact on retention characteristics, must be carefully monitored. Sensitivity, quantitation and identification of peaks with silver-ion HPLC are mainly functions of the method of detection and are thus comparable with reversed-phase HPLC procedures (Table 3).

All phospholipid molecular species chromatographic analyses are highly dependent on lipid class purification. The identity and purity of each class must be ascertained before any species characterization becomes meaningful. Only occasionally might the composition allow species of different classes in the same sample to be adequately separated by HPLC. GC and GC–MS procedures are even more dependent upon a pure lipid class as the starting material since the polar head groups are lost during derivatization making it impossible to distinguish between, for example, 18:0/20:4 diglyceride residues originating from PC or PE. However, if an LC–MS system is available, valuable species information may be obtained, often without pre-separation into individual classes.

Identification is undisputedly best performed with MS methods but positional information for the two fatty acyl groups cannot be obtained. Conventional HPLC methods require at least one fractionation of each peak and fatty acid determination by GC (or MS) to establish the identity of the molecular species. It might be a difficult task to establish the true identity of the peaks if the fraction collected is not a pure lipid class (unless an MS detector is available).

With minimal handling of the phospholipid samples and without chemical modification steps, preparation time is reduced, which reduces the risk of fatty acid oxidative degradation and other selective losses. To this end, LC–MS systems and HPLC with light-scattering detection are quite appropriate. Quantitative precision with GC methods oftentimes surpass those of LC procedures but in order to render phospholipids volatile for GC, time-consuming enzymatic and chemical reactions steps are required which may compromise accuracy.

HPLC and LC–MS methods exhibit a narrower range in which calibration curves are linear than GC with flame ionization detection. Thus, quantitative precision is dependent upon accurate calibration. Due to limited availability of defined calibration standards, detection methods generally not requiring standards for each molecular species to be determined are preferred for quantitative purposes.

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