ELSEVIER

Journal of Chromatography B, 671 (1995) 255–280

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Review

Quantitative chromatographic analysis of inositol phospholipids and related compounds

Ashok K. Singh*, Yin Jiang

Department of Veterinary Diagnostic Medicine, College of Veterinary Medicine, University of Minnesota, St. Paul, MN 55108, USA

Abstract

The metabolism of phospholipids and the mobilization of second messengers such as inositol-1,4,5-trisphosphate, 1,2-diacylglycerol (DAG) and arachidonic acid (AA) from phospholipids is commonly studied by radiolabelling phospholipids with [^{3}H]myo-inositol or [^{32}P]ATP and measuring the incorporation of radioactivity in different phospholipids or their hydrolysis products. However, for the radiolabelling method to accurately reflect changes in the compound's mass, it is essential that the tissue is labelled to isotopic equilibrium which is difficult to achieve. To circumvent the disadvantages of the radiolabelling method, several analytical procedures have been developed for the mass analysis of phospholipids and inositolphosphates (IPs). Quantitation of the mass or the radiolabelling of phospholipids is a complex multi-step procedure that involves quantitative isolation of phospholipids, fractionation of individual phospholipids and either determination of radioactivity in each component or the measurement of their mass. Phospholipids, DAG and AA are extracted from tissue sample with organic solvents such as chloroform–methanol (2:1) containing HCl or formic acid. The extract is separated by TLC, cartridge-column chromatography or HPLC on a reversed-phase column. Phospholipids are quantitated by measuring inorganic phosphate, absorption at 200 nm or mass spectrometry. Inositol phosphates are extracted with perchloric acid or trichloroacetic acid and separated by ion-exchange cartridge-column or HPLC with an ion-exchange column. IPs are quantitated by measuring inorganic phosphate or by using enzymatic reaction, metal–dye coupling, NMR or mass spectrometry.

Contents

* Corresponding author.

0378-4347/95/$29.00
SSDI 0378-4347(94)00558-3

List of abbreviations

AA	Arachidonic acid
Ca^{2+}	Calcium ions
Cer	Cerebrosides
CI	Chemical ionization
Ch	Choline
DAG	1,2-Diacylglycerol
EI	Electron-impact ionization
12-HETE	12-Hydroxyeicosatetraenoic acid
15-HETE	15-Hydroxyeicosatetraenoic acid
I{1,4}P_2	Inositol-1,4-bisphosphate
I{1,2,3,4,5,6}P_6	Inositol-1,2,3,4,5,6-hexakis-phosphate
I{1}P	Inositol-1-monophosphate
I{4}P	Inositol-4-monophosphate
I{1,3,4,5,6}P_5	Inositol-1,3,4,5,6-pentakisphos-phate
I{1,3,4,5}P_4	Inositol-1,3,4,5-tetrakisphos-phate
I{1,3,5}P_3	Inositol-1,3,5-trisphosphate
I{1,4,5}P_3	Inositol-1,4,5-trisphosphate
IPs	Inositol phosphates
LPC	Lysophosphatidylcholine
MI	Myo-inositol
NMR	Nuclear magnetic resonance
PA	Phosphatidyl acid
PAR	4-(2-phridylazo)resorcinol
PCA	Perchloric acid
PEE_2	Prostaglandin E_2
PG	Phosphatidylglycerol
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
PtdAA	Phosphatidylarachidonic acid
PtdCh	Phosphatidylcholine
PtdIns	Phosphatidylinositol
PtdIns{3,5}P_2	Phosphatidyl-3,5-bisphosphate
PtdIns{4,5}P_2	Phosphatidyl-4,5-bisphosphate
PtdIns{4}P	Phosphatidyl-4-monophosphate
PtdIns{3}P	Phosphatidyl-3-monophosphate
PtdE	Phosphatidylethanolamine
PtdSer	Phosphatidylserine
PtdIns{3,4,5}P_3	Phosphatidyl-3,4,5-trisphos-phate
Ser	Serine
SM	Sphingomyelin
SMase	Sphingomyelinase
IP_2	Total inositolbisphosphate

IP_6	Total inositolhexakisphosphate
IP	Total inositolmonophosphate
IP_5	Total inositolpentakisphosphate
IP_4	Total inositoltetrakisphosphate
IP_3	Total inositoltrisphosphate
TCA	Trichloroacetic acid

1. Introduction

1.1. Chemistry and importance of phospholipids in biological systems

The glycerophospholipids, e.g. PtdIns, PtdCh, PtdAA and PtdSer, and the sphingophospholipids, e.g. SM, are important components of biomembranes [1–3] and serve as precursors for many second messengers such as I{1,4,5}P_3, DAG and AA [4,5]. The general structure of these phospholipids is shown in Fig. 1. The 1 and 2 positions of the glycerol moiety of PtdIns, PtdCh or PtdSer are coupled with fatty acids (Fig. 1) of varying chain length, whereas the 3 position of glycerol is attached to MI, Ch or Ser. PtdAA is different from other glycerophospholipid because AA attaches to the 2 position and fatty acids attach to the 1 and 3 positions of the glycerol moiety of PtdAA (Fig. 1). The -OH group of the MI ring can be phosphorylated in any combination [4,5]. Phosphorylation of the 4 and 3 positions of MI gives PtdIns{4}P and PtdIns{3}P, respectively [4–7]. PtdIns{4}P is further phosphorylated to PtdIns{4,5}P_2 which, when acted on by PLC, produces (1) I{1,4,5}P_3 which induces the mobilization of Ca^{2+} from Ca^{2+} stores in the endoplasmic reticulum [8] and (2) DAG which activates PKC in various tissues [9]. I{1,4,5}P_3 is either phosphorylated to I{1,3,4,5}P_4 which regulates Ca^{2+} channels [10,11] or dephosphorylated to I{1,4}P_2, {1 or 4}P and MI [4]. I{1,3,4,5}P_4 is dephosphorylated to I{1,3,5}P_3, I{3,4}P_2, I{3 or 4}P and MI [4]. I{1,3,5}P_3 does not mobilize Ca^{2+}. Various animals and plants also contain I{1,3,4,5,6}P_5 and I{1,2,3,4,5,6}P_6 [12]; however, their physiological roles and metabolism are not yet fully understood. Similar to the hydrolysis of PtdIns{4,5}P_2 by PLC, PtdAA, PtdCh and SM are also hydrolyzed by specific lipases and generate second messengers. PtdAA is hydrolyzed by PLA_2 and releases AA [13]. PtdCh is hydrolyzed by PLC or PLD that produces DAG and PA [14]. SM is hydrolyzed by SMase producing ceramide, Ch or PtdCh [15]. It has been suggested that agonist-stimulation of phospholipid hydrolysis, along with releasing I{1,4,5}P_3 and DAG, also releases free fatty acids that may be playing an important role in signal transduction.

1.2. Experimental approach to the quantitation of inositol phospholipids and related compounds

The mobilization of individual phospholipids is commonly studied by labelling the phospholipid with a radionucleotide such as [^{32}P]ATP, [^{3}H]MI or [^{3}H]Ch and quantitating the formation of radiolabelled phospholipids or their hydrolysis products. Quantitation of the radiolabelling of phospholipids is a complex multi-step procedure that involves quantitative isolation of phospholipids, fractionation of individual phospholipids and determination of radioactivity in each phospholipid or its hydrolysis product [16]. In brief, total lipids, AA and DAG are extracted from tissue samples with organic solvents such as chloroform–methanol or ethyl acetate [17–19],

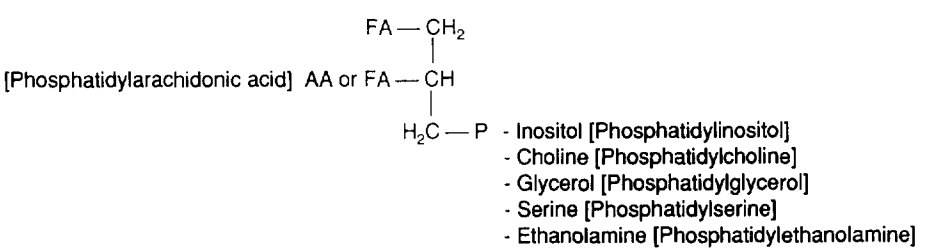

Fig. 1. Structure of phospholipids (FA: fatty acids, $RCOO^-$).

whereas IPs are extracted from tissue samples with TCA or PCA [20,21]. After extraction, phospholipids, IPs, DAG and AA are fractionated by TLC, cartridge-column chromatography, HPLC or GC. The fractions containing individual molecular species of lipids are subjected to radioactivity determination by scintillation counting. The radiolabelling method, however, has many disadvantages that can be circumvented by measuring directly the intracellular levels of the individual phospholipids or related compounds. The goal of this article therefore is to review the different extraction, chromatographic and quantitation methods available for the analysis of phospholipids and IPs in tissues.

2. Extraction of lipids from tissue samples

Total lipids are extracted from tissue samples with chloroform–methanol–0.4% HCl (2:1:0.05, v/v) as discussed previously [17]. In brief, the tissue sample is mixed with the ice-cold extraction reagent and homogenized vigorously. The homogenate is centrifuged and the organic and aqueous layers are separated. The organic layer containing the phospholipids is dried under nitrogen and used for further analysis. The aqueous layer containing the IPs is immediately neutralized to prevent acid hydrolysis of the IPs [17,22]. The extraction protocol can be simplified when extraction of only the IPs is needed. Samples are homogenized in TCA or PCA to precipitate the protein, to terminate metabolic reactions and to dissolve the IPs [22,23]. The homogenate is incubated at 4°C for 15 min and then centrifuged. The supernatant is collected and the acid is removed with either water-saturated diethyl ether for TCA or Freon and tri-N-octylamine (1:1, v/v) for PCA before analysis or storage. Previous studies have recommended the use of a neutral extraction mixture such as methanol or a mixture of phenol–chloroform and methanol for the extraction of cyclic IPs [24,25]. However, neutral extraction has several disadvantages as described previously [26]. Quantitative extraction of DAG is achieved by homogenizing the samples with methanol–chloroform–0.9% NaCl (1:3:1, v/v). The homogenate is centrifuged at 1500 *g* and the two phases are collected separately. The lower phase containing DAG is further washed with chloroform–methanol–water (1:15:16.5, v/v). AA is extracted from tissue samples by chloroform–methanol–formic acid (1:0.25:0.015, v/v) [18,19]. The organic layer is dried in a water bath at 45°C under nitrogen. The dried residue is redissolved in chloroform–methanol (2:1, v/v) and used for further analysis.

Because glycerophospholipids and its hydrolysis products are degraded very rapidly, it is essential to either extract the samples immediately after collection or freeze the samples in situ immediately after collection. In situ fixation of the samples with focused microwaves may be the method of choice for PtdIns and IPs. The frozen samples must be stored at −70°C to prevent chemical changes in the lipids. The extraction recovery can be determined by using ^{3}H-labelled standards of phospholipid, IP, DAG or AA as the external standard. Use of these compounds as an internal standard may interfere with the quantitative mass analysis of phospholipids. A recent study has demonstrated 50–90% recovery of PtdIns and IPs from brain samples [22]. IP_3 and IP_4 exhibited the lowest recovery not because of poor extraction but because of the poor quantitation method for these compounds [22].

3. Chromatographic separation of different phospholipids

The next step in the quantitative analysis of phospholipids is the separation of lipid compounds from non-lipid contaminations and then the separation of the individual phospholipids by using different chromatographic procedures. The most commonly used method is TLC in which the extract is applied on a TLC plate, the plate is developed in an appropriate solvent and the developed plate is sprayed with a color reagent such as 2′7′-dichlorofluorescein or a mixture containing 37% formaldehyde and concentrated sulfuric acid (3:97, v/v) [27,28]. Phospholipids

can be recovered from silica by elution with appropriate solvents such as chloroform–methanol (2:1, v/v) for phospholipids or 0.1% HCl for IPs. The TLC method is simple and rapid, although it exhibits poor recovery of minor components. Alternative techniques for the separation of lipids are cartridge-column chromatography using Sephadex, silicic acid, ion-exchange cellulose or immobilized neomycin columns, HPLC and GC. As discussed earlier, glycerophospholipids contain fatty acids attached to its glycerol moiety. It may be necessary to remove fatty acids (Fig. 2) from phospholipids when (1) the chromatographic separation of phospholipids is based on the structural differences in their inositol head group [29] and (2) fatty acids interfere with the chromatographic separation [30]. It should be noted that the deacylation process may hydrolyze the glycerophosphoester backbone, thus resulting into an artificial increase in inositol phosphates. Therefore, care should be taken to minimize this process. A deacylation procedure using monomethylamine has been reported to minimize hydrolysis of the glycerophosphoester bond [31]. It is also essential to remove water-soluble nucleotides by using charcoal extraction because nucleotides such as ATP coelute with I{1,4,5}P_3 in ion-exchange cartridge-column or HPLC.

Despite the development of numerous chromatographic procedures for the separation of lipids in tissues, complete separation of all molecular lipid species in a crude extract can not be achieved by any one of these methods. Common practice therefore is that the lipid extract is first separated into different groups of phospholipids by using column chromatography and then each group is further separated into its individual lipids by TLC, column chromatography or HPLC. In this section, the procedures, advantages and disadvantages of the different chromatographic procedures will be discussed.

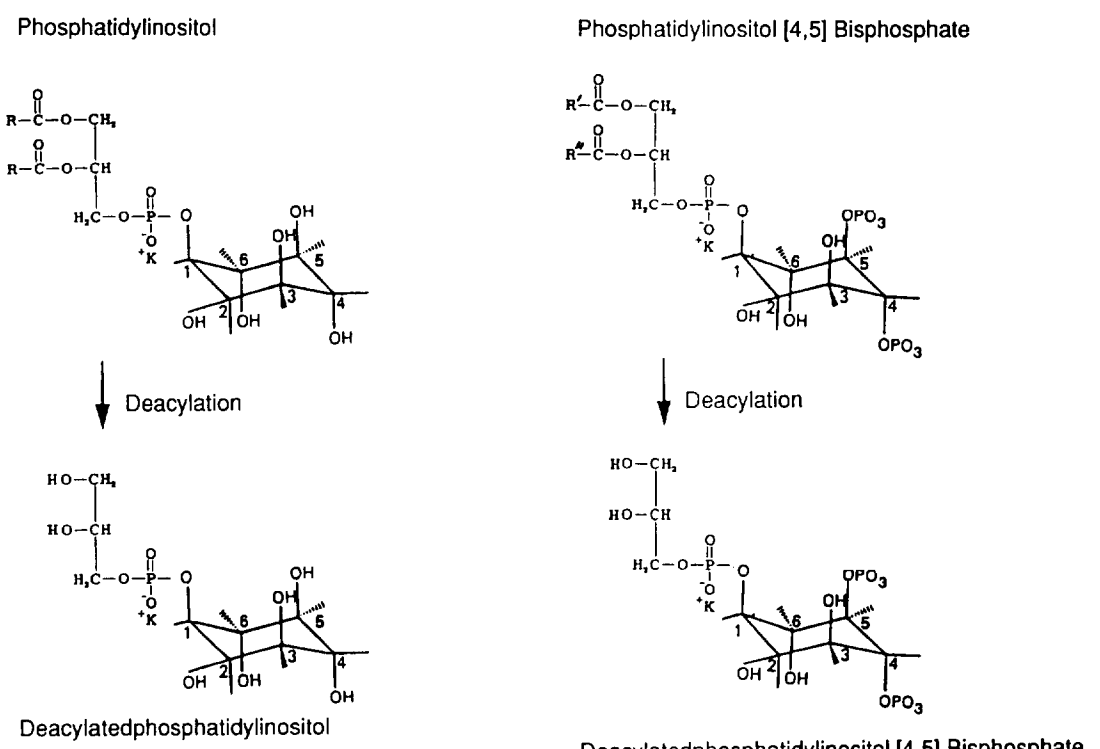

Fig. 2. Structures of acylated and deacylated PtdIns and PtdIns{4,5}P_2. As shown in the figure, phosphorylation of PtdIns alters its fatty acid composition (RCOO, R'COO and R"COO are different fatty acids).

3.1. Thin-layer chromatography

Crude lipid extracts or mixtures containing different groups of phospholipids are further separated into their individual lipids mostly by TLC. The commonly used TLC plates are glass plates coated with silica gel H or alumina. Coating of the silica plate with magnesium silica or oxalate has been reported to improve the separation of phospholipids [32,33]. Plastic-based silica plates are not suitable for solvents containing chloroform. Previously investigators have used double-bed TLC plates with the bottom half of the plate embedded with alumina or the weak anion exchanger Cellex PAB and the top half embedded with silica for the separation of drugs and other compounds [34,35]. The dual coated plates provide excellent resolution although these plates can not be used for two-dimensional TLC.

For TLC analysis, the lipid extract is evaporated to dryness under nitrogen and the dried residue is redissolved in 50 μl of chloroform–methanol (1:1, v/v). The concentrated extract is then spotted at one end of the activated plate under 25–30% humidity which is necessary for complete separation of many phospholipids such as PtdIns and PtdSer. The spot is dried and the plate is developed in appropriate solvent mixtures. The solvent mixture diffuses through the solid adsorbent phase such that the different compounds present in the lipid extract migrate at different rates, thus allowing separation of the individual phospholipids [36–38]. The migration of phospholipids on TLC plates depends upon the properties of the solid phase and the solvent used [39,40] as shown in Table 1. The separation of phospholipids is improved by two-dimensional chromatography in which a TLC plate is first developed in one direction for up to a preset level and then the plate is dried and developed again in another solvent mixture at an angle perpendicular to the first development. After the solvent has migrated to a preset level, the plate is removed from the tank, dried and sprayed with a color reagent. As shown in Fig. 3, two-dimensional chromatography separated several phospholipids with good resolution. In two-dimensional TLC, however, care should be taken not to expose the plate to air and to avoid mixing of the two solvents during changing of the plate from one solvent to another. The dried plates are sprayed with the color reagents. The commonly used reagents are (1) 2′-7′-dichlorofluorescein (0.1%) in methanol–water (97:3, v/v) which reacts with certain phospholipids and exhibits fluorescence, (2) a mixture containing 37% formaldehyde and conc. sulfuric acid (3:97, v/v) which is sprayed on the plate and then heated at 180° for 30 min and causes the lipid compounds to appear as black charred spots, or (3) a mixture containing 3% copper acetate in 8% phosphoric acid which makes phospholipids to appear dark brown.

3.2. Cartridge-column chromatography

Sephadex, ion-exchange cellulose, silicic acid, immobilized neomycin and C_{18} Sep-Pak columns have been successfully used for the separation of phospholipids from tissue extracts. Sephadex columns can be used to remove water-soluble non-lipid contaminations from lipids and for the separation of gangliosides from the other phospholipids [41]. Silicic acid columns are excellent for separating neutral lipids from polar lipids or glycolipids with other lipid groups [42]. Ion-exchange cellulose columns such as DEAE- and TEAE-cellulose columns provide excellent separation of lipid classes without cross-contamination [43]. DEAE-cellulose columns are more effective in separating polar lipids than TEAE-cellulose columns which are more effective in separation weakly acidic lipids. Rouser et al. [28] have developed extensive protocols for the stepwise elution of different lipids from DEAE or TEAE columns. Acidic lipids such as fatty acids are eluted from DEAE columns with weekly-acidic solvent but strongly acidic lipids are eluted from the columns by chloroform–acetic acid–ammonia (3:1:0.1, v/v) containing salt such as ammonium or potassium acetate. PtdCh exhibits reversible binding with DEAE and is eluted easily with chloroform–methanol (9:1, v/v). Nonionic lipids are separated from DEAE columns with chloroform, hexane or hexane–di-

Table 1
Separation of phospholipids by TLC [27,28,36–39].

Solvent system	Solvent composition	Comments
A		
Chloroform–methanol–4 *M* ammonia	60:40:10	Oxalate coated plate separates PtdIns, PtdOH and PtdInsP
B		
Chloroform–methanol–acetone–16 *M* NH_4OH, then developed in:	70:45:10:10	
Chloroform–methanol–16 *M* ammonia-water	36:28:2:6	Redeveloping separates PtdInsP from PtdIns[4,5]P_2
HCl fumes		HCl fumes essential for the separation of Ptdins but overexposure may hydrolyze fatty acids
Developed at 90° to the first front in chloroform–methanol–acetone–acetic acid–0.1 *M* ammonia	140:60:55:4.510	
C		
Methyl acetate–2-propanol–chloroform–methanol–25% KCl in one direction	25:25:25:10:9	Good separation of polar lipids
Chloroform–acetone–methanol–acetic acid–water in the other direction	10:4:2:2:1	
D		
Chloroform–methanol–28% ammonia in one direction	65:25:5 or 65:85:5	Excellent separation of polar lipids. Humidity of the spotting sample is critical for the separation of PtdIns from PtdSer
Chloroform–acetone–methanol–acetic acid–water in the other direction	3:4:1:1:0.5	
E		
Hexane–diethyl ether or hexane–diethyl ether–acetic acid in one direction	90:10 80:20:2	Neutral and least polar lipids
Chloroform–methanol–28% ammonia in the other direction	95:5:.8	
F		
Chloroform–methanol–ammonia in one direction	65:85:5	Separation of polar lipids. Provided better separation of Cer and Su but poor separation of PtdCh and PtdSer
Chloroform–acetone–methanol–acetic acid–water in the second direction	4:3:1:1:0:5	

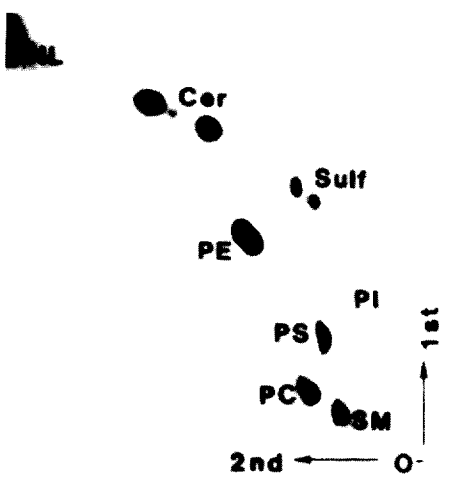

Fig. 3. Separation of lipids by two-dimensional TLC. White matter samples were dissected out and extracted with chloroform–methanol (2:1, v/v) as described previously [17]. The lipid extract was dried under nitrogen. The dried residue was redissolved in hexane–propanol and spotted on a HPTLC plate. The plate was developed in one direction using methyl acetate–propanol–chloroform–methanol–0.25% aqueous KCl (25:25:25:10:9, v/v) and in the other direction using hexane–diethyl ether–acetic acid (80:20:2, v/v). The plate was removed and dried. The dried plate was sprayed to saturation with 3% copper acetate in 8% phosphoric acid. Phospholipids were visualized after charring the plate for 20 min at 160°C. Reprinted from Ref. [27] with permission. PE = phosphatidylethanolamine, PI = phosphatidylinositiol; PS = phosphatidylserine, PC = phosphatidylcholine, Sulf = sulfatide, NL = neutral lipids.

ethyl ether. Although a recent study has described the use of a C_{18} Sep-Pak column for the separation of lipids, the column is suitable mostly for the separation of neutral lipids [44]. The immobilized neomycin columns are excellent for separating the different inositol phospholipids [45–51] as shown in Fig. 4 [46]. The different inositol phospholipids are eluted from the neomycin columns by increasing the concentration of ammonium formate: PtdIns, PtdInsP and $PtdInsP_2$ eluting at 20, 400 and 1000 m*M* ammonium formate, respectively [46].

As discussed earlier, none of the above columns is effective in separating from crude tissue extracts the individual phospholipids in pure form. Therefore, lipid extracts are processed by several chromatographic steps. For this purpose, the extract is first subjected to Sephadex chromatography for separation of the lipids from the nonlipids in the extract. The lipid fraction is then poured onto a DEAE column that is eluted with different solvents in the following order: (1) chloroform to elute neutral lipids, (2) a mixture of chloroform–methanol (9:1) and acetic acid (0.02%) to elute fatty acids, Cer, PtdCh, LPC and SM; (3) chloroform–methanol (7:3, v/v) to elute PtdE, (4) methanol to remove salt and (5) chloroform–methanol–ammonium salt to elute PA, PtdIns and PG [22,28]. The individual fractions are concentrated to 100 μl at 45°C under nitrogen and then the concentrated residue is further separated by cartridge-column chromatography, TLC, HPLC or other chromatographic procedures. A recent study has shown that Sep-Pak cartridges (Accel Plus QMA, Cat. No. 20815, Millipore, Bedford, MA, USA) provide excellent separation of PtdCh, PtdIns, PtdInsP and $PtdInsP_2$ from DEAE eluate as shown in Fig. 5 [22].

3.3. High-performance liquid chromatography

As discussed earlier, the separation of lipids from crude extracts by TLC and ion-exchange columns involves multiple steps of sample preparation that may be time-consuming. HPLC has been reported to be a simple and sensitive method for the separation of lipids in crude extracts [52,53]. The HPLC system commonly used for the separation of phospholipids consist of two pumps, a solvent delivery system, a reversed-phase column and a detection system such as UV detector, mass spectrometer or radiometric detector. UV detection of underivatized phospholipids eluted from HPLC columns is achieved by measuring the absorption in the 200-nm region [52]. Since many solvent mixtures are not transparent in the 200-nm UV range, the use of this method limits the choice of solvent systems. Recent studies have shown that acetonitrile–methanol–water [53] and hexane–2-propanol–water [54,55] solvent systems are transparent in the 200-nm region and provide excellent separation of phospholipids. Fig. 6 demonstrates the elution order of plasma membrane lipids as determined by HPLC and UV detec-

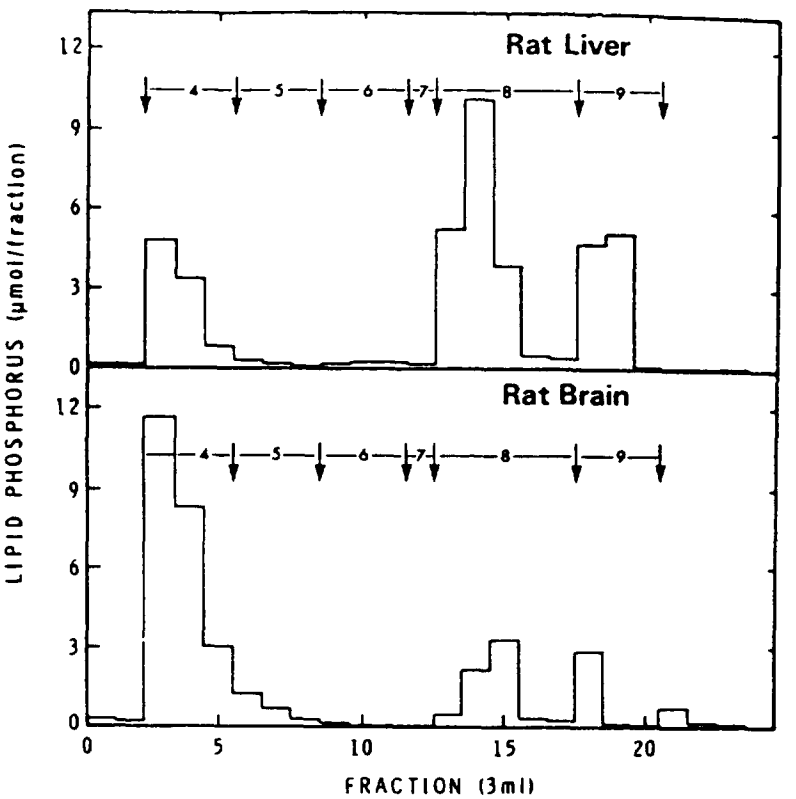

Fig. 4. Fractionation of acidic phospholipids on immobilized neomycin column. Brain and liver samples from rats were extracted with chloroform–methanol (1:1, v/v). The samples were centrifuged and the extract was collected. The tissue sample was re-extracted with chloroform–methanol (2:1, v/v). The sample was again centrifuged and the extract was separated and pooled with the previous extract. The combined extracts were diluted with 4 volumes of chloroform containing 0.2 ml of 1 *M* HCl. The organic phase was collected and dried under nitrogen. The dried residue was redissolved in chloroform–methanol (2:1, v/v) and the entire extract was poured onto a neomycin sulfate column. The column was sequentially washed with 4 to 6 volumes of chloroform–methanol (1:1, v/v), chloroform–methanol (2:1, v/v), chloroform–methanol–88% formic acid (300:600:1, 10:20:1, 10:20:1 and 5:10:1, v/v), chloroform–methanol–water (5:10:2, v/v) and ammonium formate 20–40 m*M*, 100–200 m*M*, 400–600 m*M* and 1 *M*. The amount of radioactivity was determined in each fraction. PtdIns, PtdIns[P] and PtdIns[P_2] eluted in steps 8 to 11. Reprinted from Ref. [46] with permission.

tion. HPLC using an acetonitrile- or hexane-based mobile phase allows the direct detection of phospholipids without prior cleanup of the crude extract, thus making these methods simple and rapid. However, these methods do not effectively separate the different inositol phospholipids. Recently, Nakamura et al. [56] have developed an HPLC procedure that specifically analyzes PtdInsP and PtdInsP_2 in the brain. In this method, the lipid extract is first derivatized with 9-anthryldiazomethane to produce (9-anthryl)PtdInsP and di(9-anthryl)PtdInsP_2 and then the derivatized sample is separated by HPLC with UV detection at 245 nm. PtdCh, PtdIns, PtdSer, PtdE and SM are not derivatized with this reagent and, therefore, do not interfere with the assay [57]. This method is more sensitive than that using the underivatized UV detection method for the analysis of inositol phospholipids. Previous studies have described a novel HPLC method that uses thermospray mass spectrometry for the detection of phospholipids in crude extracts [58,59]. In this method, phospholipids are separated on a reversed-phase column with methanol–hexane–0.1 *M* ammonium acetate as the mobile phase. The column

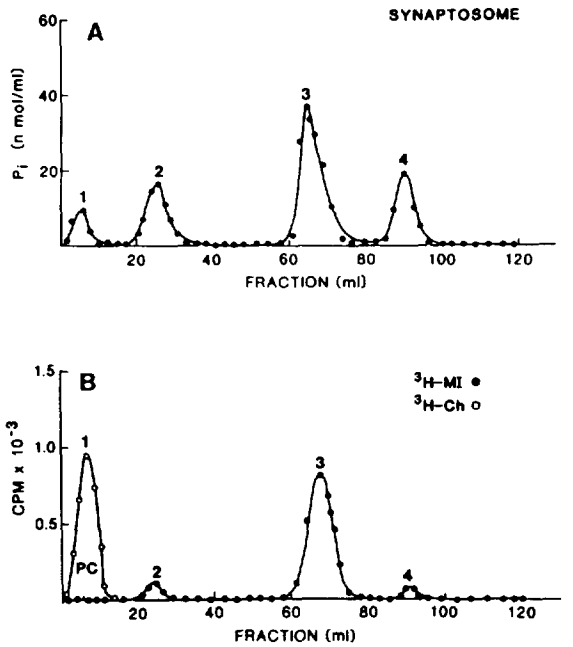

Fig. 5. Fractionation of PtdCh, PtdIns, PtdIns{4}P and PtdIns{4,5}P_2 by Sep-Pak column. Synaptosomes were labelled with [^{3}H]MI and the loaded samples were extracted for total lipid. The lipid extract was concentrated under nitrogen and then applied onto a DEAE-cellulose column and different phospholipids were eluted from the column. The fractions containing PtdCh and inositol phospholipids were pooled and concentrated under nitrogen. The concentrate was applied onto the Sep-Pak column and eluted with a gradient of 0.5–99.5% of ammonium acetate. A clear separation of PtdCh, PtdIns, PtdIns{4}P and PtdIns{4,5}P_2 was obtained. Reprinted from Ref. [22] with permission.

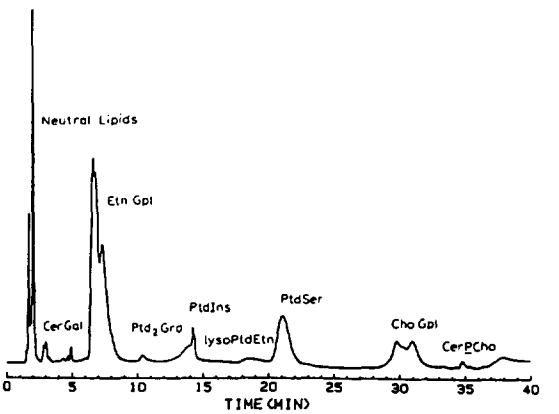

Fig. 6. Elution of bovine brain plasma membrane lipids with HPLC. The membrane samples were extracted 3 times with 1:18 (w/v) ratio of hexane–2-propanol (3:2, v/v). The extracts were pooled and concentrated to dryness under nitrogen. The dried residue was redissolved in hexane–2-propanol (3:2, v/v) and injected onto the HPLC system consisting of 2 pumps, a solvent controller and a UV detector. Phospholipids were separated on a Dupont Zorbax SIL (25 cm × 4.6 mm I.D., 5 μm) column. Two solvents (solvents A and B) were used as the mobile phase. Solvent A was hexane–2-propanol (3:2, v/v) and solvent B consisted of 27.5% water and 72.5% solvent A. The two solvents were programmed as follows: initial 50% each of A and B, increasing B to 78% in 9 min, increasing B to 100% in 23 min and resetting of the initial values in 2 min. The flow-rate was 1.5 ml/min and the UV detector was set at 205 nm. This program separated neutral lipids from acidic lipids but did not resolve the individual inositol phospholipids. Reprinted from Ref. [52] with permission.

effluent is introduced into a mass spectrometer via a Vestec thermospray interface. The mass spectrum of each phospholipid is determined. A typical mass spectrum of PtdIns obtained by HPLC–MS analysis is shown in Fig. 7. Fig. 7b demonstrates that the ion at m/z 198 corresponds to inositol-NH_3 that may be characteristic of all inositol phospholipids. As shown in Fig. 7a, PtdIns may exhibit multiple peaks depending on the composition of the fatty acids interacting with the phospholipid. Deacylation of the phospholipids before analysis may improve the analysis of phospholipids. The HPLC–MS method has potential for quantitating the concentration of individual lipids by measuring selected ions.

The HPLC methods described above are designed to separate the inositol phospholipids that exhibit phosphorylation at 4-OH position of the inositol ring (such as PtdIns[4}P and PtdIns{4,5}P_2). Recent studies have shown that certain cells also contain a novel phosphoinositide kinase that phosphorylates the 3-OH position of the inositol ring, thus producing PtdIns{3}P, PtdIns{3,4}P_2 and PtdIns{3,4,5}P_3 [60]. These 3-P phospholipids are different from 4-P phospholipids since the former phospholipids do not act as substrate for PLC [61]. These phospholipids were not detected previously because they are present only in very low amounts and co-migrate with the 4-P inositol phospholipids in the conventional chromatographic methods. Recently Auger et al. [61]

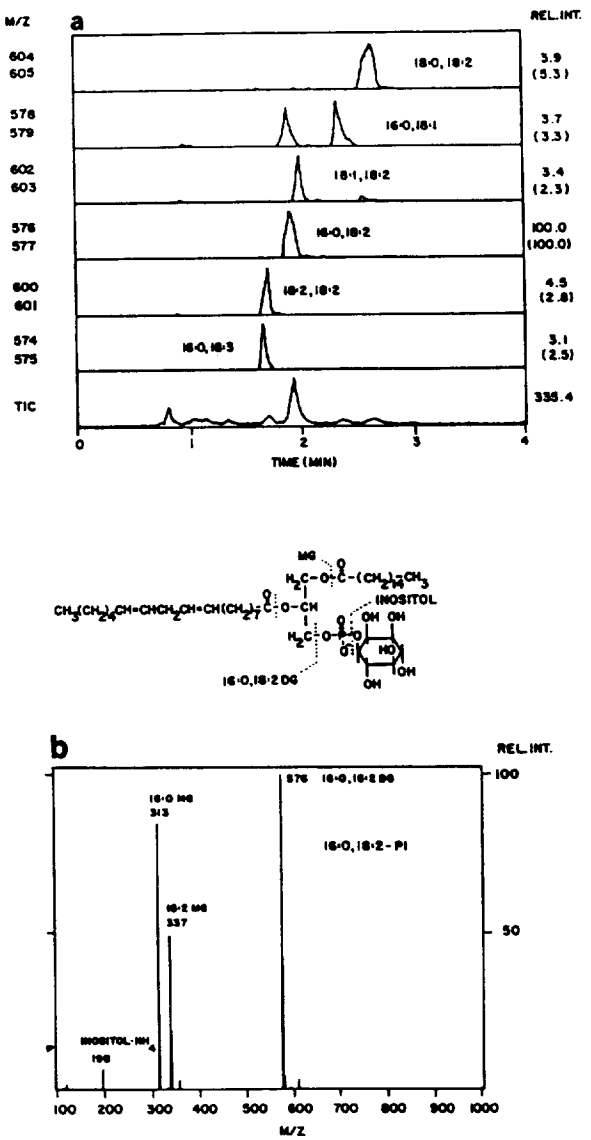

Fig. 7. Analysis of PtdIns by HPLC–MS. (a) Separation and selected-ion monitoring of plant PtdIns and (b) thermospray mass spectrum obtained from the 16:0,18:2 peak. The tissue samples were extracted for total lipids and the extract was concentrated under nitrogen. The concentrated extract was separated by HPLC on an Altex Ultrasphere-ODS (7.5 cm × 4.6 mm I.D., 3 μm particle size) column. The mobile phase was methanol–hexane–0.1 *M* ammonium acetate in water (500:25:25, v/v) and the flow-rate was 1 ml/min. The HPLC eluate was introduced into a mass spectrometer equipped with a Vestec thermospray interface. At least 6 molecular species of PtdIns were detected based on the differences in the chain length and the saturation level of the fatty acid attached to it. Reprinted from Ref. [58] with permission.

developed an ion-exchange HPLC method for the identification of PtdIns, PtdIns{3}P, PtdIns{3,4}P_2 and PtdIns{3,4,5}P_3. The method requires prior deacylation of the phospholipids because the HPLC method separated the phospholipids based on the structural differences in the inositol head group [61].

4. Chromatographic separation of inositol phosphates

4.1. Thin-layer chromatography, paper chromatography and electrophoresis

Previous studies have used silica-gel or PEI-cellulose TLC plates for the separation of IPs [62–64], although the methods are not very sensitive and exhibit poor resolution. Paper chromatography provides better separation of IPs but exhibits poor sensitivity [64–67]. Electrophoresis on Whatman No. 1 paper or cellulose TLC plates provides relatively rapid separation of IP polyphosphates in brain extracts [68]. Electrophoresis effectively separates IP isomers that ion-exchange columns can not [68]. However, the loading capacity of electrophoresis paper is very small. These observations suggest that TLC, paper chromatography or electrophoresis may not be suitable for the routine analysis of IPs in small quantity of tissue samples.

4.2. Cartridge-column chromatography

Anion-exchange cartridge columns such as Dowex formate, AG 1 X-8 formate or DEAE-cellulose columns provide excellent separation of IPs in biological samples. The commonly used method involves deproteinization of the samples with TCA or PCA, neutralization of the acid and stepwise elution of IP, IP_2, IP_3 and IP_4 with increasing concentration of ammonium formate in dilute formic acid [69,70]. Ammonium formate elutes IPs because formate has greater affinity than phosphate for the resin. However, a very high concentration of formate may be needed for the elution of IP_3 and IP_4 which may exhibit some cross-contamination [70]. In

another study, polyinositol phosphates were eluted from an anion-exchange resin by a stepwise gradient of ammonium sulfate [71] that provided excellent separation of IP, IP_2, IP_3 and IP_4 as shown in Fig. 8. Since sulfate has a greater affinity for the resin than either formate or phosphate, the elution of IPs is achieved at a relatively low ionic strength [71]. A recent study has described the use of Sep-Pak columns for the separation of IPs [22]. In this method, PCA extracts from tissue samples are poured onto a Sep-Pak column and the IPs are eluted by using a linear gradient of TEAB from 10 to 100% [22]. As shown in Fig. 9, this method separated PtdCh and different inositol phospholipids in small

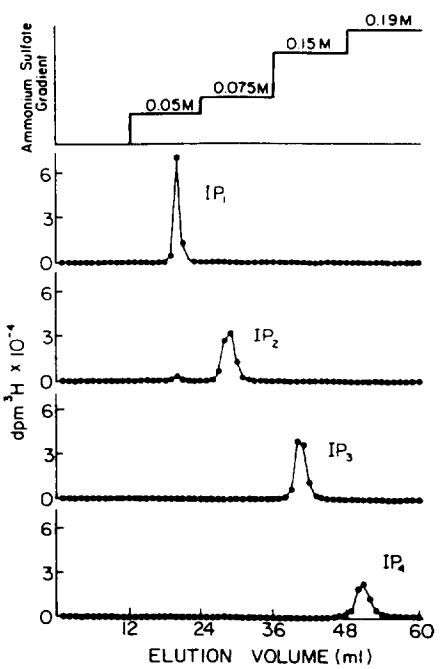

Fig. 8. Elution pattern of IPs applied to an anion-exchange resin (AG1-X8) and eluted with ammonium sulfate. The myocyte samples were loaded with [^{3}H]MI and the loaded samples were extracted twice with TCA. The TCA extracts were pooled and washed with water saturated diethyl ether. The extract was then lyophilized to dryness. The dried sample was resuspended in 2 ml of Tris buffer (0.01 *M*, pH 8.5), neutralized with $NaHCO_3$ and applied to the resin (Bio-Rad AG1 X8). IP, IP_2, IP_3 and IP_4 were eluted with 12 ml of 0.05, 0.075, 0.15 and 0.16 *M* ammonium sulfate, respectively. This method provides excellent separation of IPs. Reprinted from Ref. [71] with permission.

sample size. The ion-exchange methods described above, although providing excellent separation of IPs, have several disadvantages, e.g. (1) the methods do not resolve different IP isomers or IP_5 and IP_6, and (2) the interfering compounds are not identified by these methods. Bartlett [72] has developed an improved ion-exchange method that permits the separation of poly-IPs from IP to IP_6 in blood samples. In this method, the red blood cells are extracted with PCA and the PCA extract is neutralized with KOH. The neutralized extract is poured onto a Dowex formate column and the IPs are eluted with a linear gradient of 0 to 5 *M* ammonium formate followed by a linear gradient of 0 to 1 *M* HCl. Fractions of 5 to 10 ml are collected and assayed for IPs by measuring the phosphate concentration [73]. This method provided excellent separation of IP, IP_2, IP_3, IP_4, IP_5 and IP_6. However, the method is not suitable for routine analysis of IPs since it requires large sample volumes and large amounts of elution reagents. Recently, Christensen and Harbak [74] described an ion-exchange method that is suitable for the separation of poly-IPs in small tissue samples (Fig. 10).

4.3. High-performance liquid chromatography

One of the major disadvantages of ion-exchange chromatography is its inability to separate IP isomers. Previous studies have shown that poly-IPs exist in several isomeric forms that differ in their metabolism and biological activity [4,5]. Therefore, ion-exchange chromatography may not be suitable for studying IP metabolism or the possible relationship between IPs and their biological activity. HPLC may be more suitable for this purpose because it provides excellent separation of the IP isomers. The early HPLC methods were developed especially for the separation of I{1,4,5}P_3 from I{1,3,5}P_3 [75,76] or the isolation of different IPs [77,78]. However, numerous HPLC methods using different columns, solvent mixtures and modes of detection are presently available for the analysis of IPs (Table 2, Figs. 11–16). The choice of HPLC method will depend upon the objectives

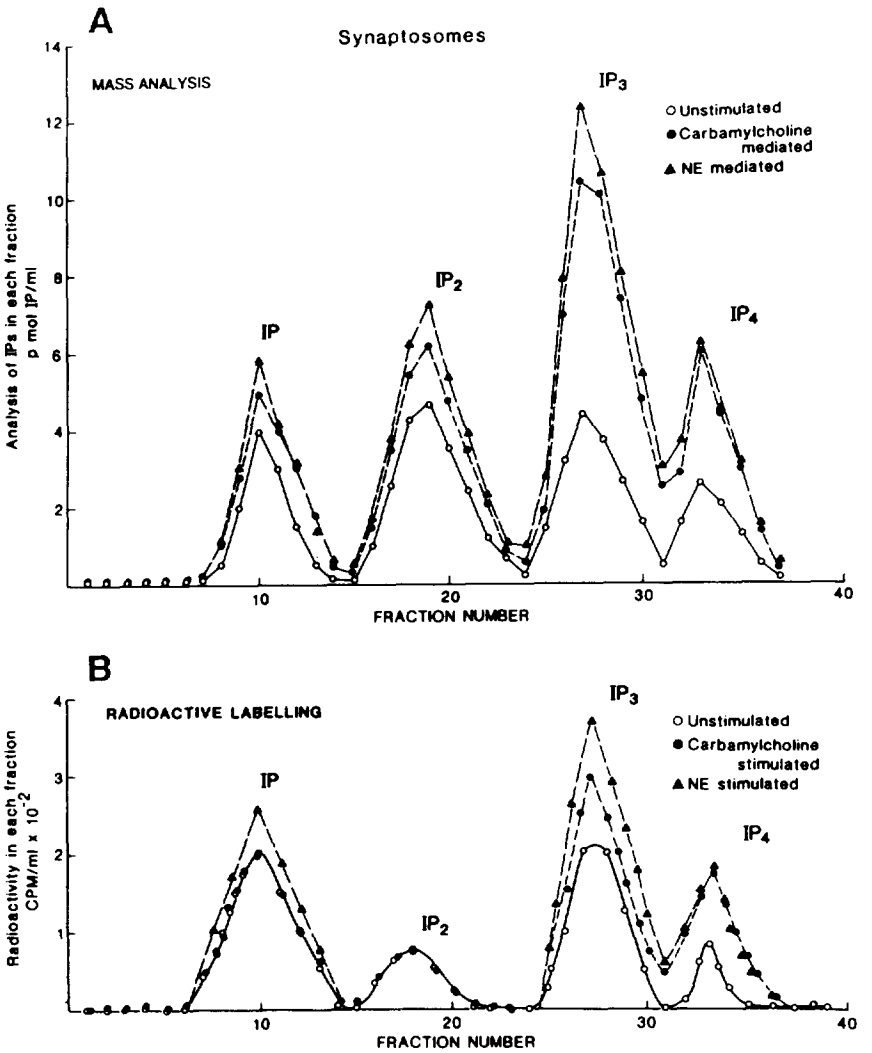

Fig. 9. Application of the Sep-Pak column in the separation of IPs in synaptosomes. Synaptosomes were loaded with [^{3}H]MI and then incubated with Ringer's buffer alone or the buffer containing carbamylcholine or NE. The stimulated and unstimulated samples were precipitated with TCA and centrifugation. The clear supernatant was collected and neutralized with 10 *M* KOH, Then the sample was applied to a Dowex-50W column and the eluate was collected. The column was washed with 5 column volumes of water and the eluates were pooled. The combined eluate is lyophilized overnight. The dried residue is redissolved in deionized water and poured onto a Sep-Pak column. Individual IP was eluted by using a linear gradient of TEAB (1 *M*) from 10% (90% water) to 90% (10% water) in 60 min. The eluate was collected and subjected to the analysis of IPs or scintillation counting. This method provides good separation of IPs, although the isomers are not separated. Reprinted from Ref. [22] with permission.

of the experiment and the questions asked. If one wishes to study changes in total IPs, then ion-exchange chromatography or simple HPLC methods as described by Reed and Bellerache [79], or Mathews et al. [80] should be used. However, if one wants to study IP metabolism, regulation of Ca^{2+} mobilization by I{1,4,5}P_3 or the redistribution of Ca^{2+} by I{1,3,4,5}P_4, then methods that provide effective separation of the IP isomers will be more suitable. HPLC methods

Table 2
Separation of IPs by different HPLC systems.

Column	Mobile phase	Detection	Fig.	Comments	Ref.
Partisil SAX anion-exchange	Ammonium formate (pH 3.7) and water gradient 0–1 *M*	Radioactivity	–	Separation of IPs No isomer separation	79
Partisil SAX	Water–methanol (95:5) and 0–95% linear gradient of 1 *M* citrate in methanol	Phosphorus	–	Separation of IPs but not isomers	80
Nucleosil 5/5B	0–350 m*M* gradient of KH_2PO_4 containing 350 m*M* KCl at pH 2.7	Radioactivity	–	Separation of IPs. ATP not separated from IP_3	81
Mono-1 AE	0.2 m*M* HCl containing 9–18 μM transition metal and 0.4 m*M* HCl containing 14–28 μM transition metal	Metal–dye	11	Rapid separation of IP isomers with good resolution	82 83
ASP 4 IE	4–37% non-linear gradient of cyanophenol in water	Conductivity	–	Simultaneous separation of IPs, anions and nucleotides	84
Partisil SAX	0–2 *M* non-linear gradient of ammonium formate, pH 3.7, adjusted with phosphoric acid	Radioactivity	12	Separation of IP_3 isomers	85
Mono Q HR or Aminex A27	Buffer A: 0.1 m*M* $ZnSO_4$ and 5 m*M* Hepes, pH 7.4; Buffer B: 1 *M* sodium sulfate, 5 m*M* Hepes and 0.1 m*M* Zn^{2+}	Phosphate	–	Poor resolution of IPs. Isomers not separated	86
Dionex AS-7	100 m*M* Sodium hydroxide and 100 m*M* sodium acetate	Amperometeric	–	Designed for the separation of I{1}P from I{4}P	87
Partisil SAX	0–1.75 *M* stepwise gradient of $(NH_4)H_2PO_4$	Radioactivity	13	Separation of IP isomers	88
Partisil SAX	0–100% gradient of 0.5 *M* $(NH_4)H_2PO_4$ at pH 3.2	Metal–dye, NMR	14	Separation of IP isomers	89
PartiSphere WAX	0–95% of 0.5 *M* $(NH_4)H_2PO_4$ at pH 3.2		15	Separation of 4 isomers of IP_5	
Mono Q HR	A: 0.1 m*M* $ZnSO_4$, 0.1 m*M* EDTA, 10 m*M* Hepes, pH 7.4; B: buffer A containing 0.5 *M* Na_2SO_4; 0–100% linear gradient of B	UV	–	Rapid separation of IPs but not isomers	90
Partisil SAX	0–100% non-linear gradient of ammonium phosphate, pH 3.8	Radioactivity	16	Excellent separation of IP isomers	91

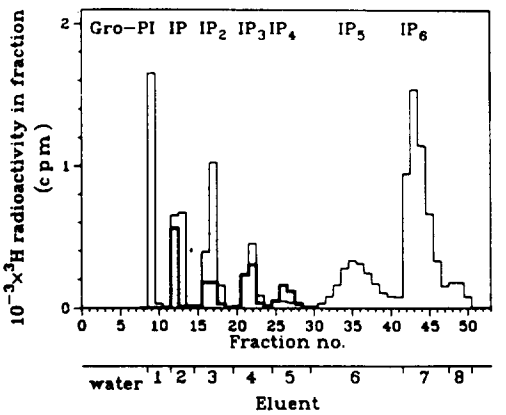

Fig. 10. Separation of IPs including IP_5 and IP_6 on anion-exchange column. Ehrlich ascites tumor cells were labelled with [^{3}H]MI and extracted with TCA. The TCA extract was washed with water-saturated diethyl ether and then mixed with borate buffer (7.8 m*M*, pH 8.0) containing EDTA (12.5 m*M*). The samples were applied on a Dowex 1-X8 (200–400 mesh) column and eluted stepwise with ammonium formate (0.06 *M*, 9 ml; 0.15 *M*, 9 ml; 0.3 *M*, 15 ml, 0.65 *M*, 15 ml; 0.80 *M*, 15 ml; 0.85 *M*, 44 ml; 1.2 *M*, 24 ml and 2 *M*, 12 ml). The flow-rate was set at 1 ml/min and 1-ml fractions were collected. Each fraction was subjected to scintillation counting. The thick line represents commercial ^{3}H-labelled I{1}P, I{1,4}P_2, I{1,4,5}P_3 and I{1,3,4,5}P_3 standards. Reprinted from Ref. [74] with permission.

that do not effectively separate ATP from I{1,4,5}P_3, such as the one described by Brammer and Weaver [81], are not suitable for experiments that use radiolabelling with [^{32}P]ATP.

The commonly used HPLC methods separate IPs on ion-exchange columns by using gradients of aqueous mobile phases. The separation of IPs depends upon the type and size of the column, the composition of the solvent mixture and the gradient of the solvents. In general (1) short columns provide more rapid and sharper separation of IPs than large columns, (2) shallower gradients provide better separation but longer chromatographic run-times than steep gradients, (3) stepwise gradient increments provide better resolution of IP isomers than linear increments and (4) solvents with pH < 4 provide sharp separation of IPs. Exposure to low pH may hydrolyze IPs and produce false values if inorganic phosphate is being detected for the identification of the IPs. Most HPLC procedures use linear or stepwise gradients of ammonium formate, ammonium acetate and/or ammonium phosphate as the mobile phase. Irvine et al. [76] identified IPs by adding a mixture of AMP, ADP and ATP to the extracts that served as markers for IP, IP_2 and IP_3, respectively. This was done because (1) IPs do not absorb UV or visible light but nucleotides do and (2) these nucleotides elute close to the respective IPs. Several studies have demonstrated direct detection of IPs by using metal–dye [82], amperometric [83] and conductivity detection [84].

4.4. Gas chromatography

Although gas chromatography has been used for the direct analysis of I{1}P [92], GC columns do not effectively separate the different molecular species of IP. Therefore GC is not suitable for the direct analysis of poly-IPs. In previous studies a GC method for the analysis of MI has been developed that can also be used for the indirect analysis of IPs [93,94]. The method involves separation of IPs in crude extract, dephosphorylation of individual IP into MI by using alkaline phosphatase and subsequent analysis of MI by gas chromatography. MI is detected either by a flame-ionization detector [93] or by mass spectrometry [94]. Fig. 17 shows the chromatographic separation of natural and d_6-labelled MI by using selective-ion monitoring GC–MS. A major disadvantage of the GC procedures is that IPs must be extracted in a form that can be dephosphorylated by alkaline phosphatase. Rittenhouse and Sesson [94] achieved this by eluting IPs with ammonium formate and then subjecting the IPs to a second ion-exchange chromatography using LiCl. This method provided poor and variable recovery because the use of ethanol to remove the excess LiCl caused large losses of IPs. Heathers et al. [71] and DaTorre et al. [93] circumvented this problem by using ammonium sulfate for the elution of IPs from the resin.

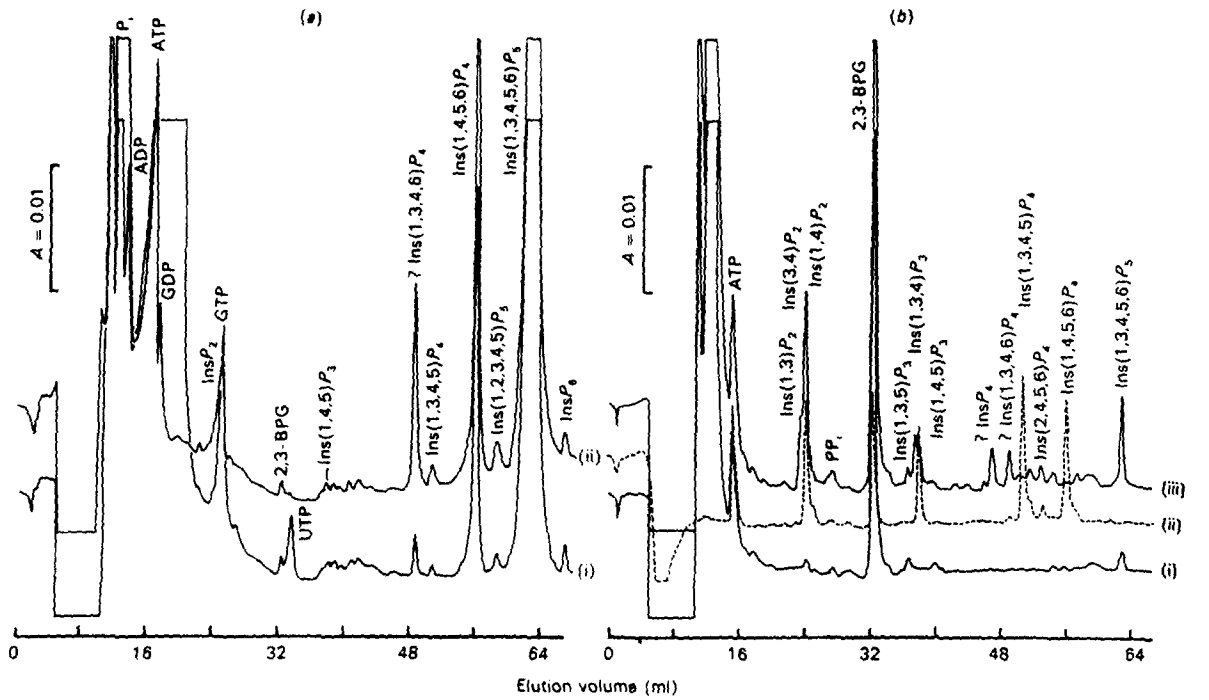

Fig. 11. HPLC analysis of the different IP isomers including IP_5 and IP_6 using metal–dye detection. Blood or platelet samples were extracted by homogenizing the samples with ice-cold PCA (0.5 *M*). The samples were centrifuged and the clear supernatant was collected. The pH of the extract was adjusted to pH 5 with KOH. Nucleotides were removed from the sample by using acid-treated Norit A charcoal (20% suspension w/v prepared in 0.1 *M* NaCl in 50 m*M* sodium acetate, pH 4). The charcoal-treated extract was injected onto the HPLC system equipped with gradient pumps, UV detector and a Mono Q column (15 cm × 0.5 cm I.D.). The two mobile-phase solvents were 9 μM YCl_3 (A) and 14 μM YCl_3. The initial gradient was A:B (1:2) and the percentage of solvent B was increased gradually to 95%. The HPLC eluates were subjected to post-column treatment with PAR. IPs were detected at 520 nm. Chromatogram (a) shows the composition of IPs in avian normoxaemic (i) and anoxaemic (ii) blood. Chromatogram (b) shows the composition of IPs in platelets before (i) and after (ii and iii) secretion. This method provides rapid separation of IP isomers with excellent resolution. This method is suitable for mass analysis of IPs. Reprinted from Ref. [82] with permission.

5. Chromatography of diacylglycerol and arachidonic acid

DAG is commonly measured by extracting samples with chloroform–methanol, drying the chloroform layer and incubating the dried residue with [γ-^{32}P]ATP and DAG kinase, separating the product (1-stearoyl-2-arachidonoyl-*sn*-glycerophosphate) by TLC and determining radioactivity in the product [95,96]. DAG is separated from other lipids either by TLC (Fig. 18) or by HPLC (Fig. 19) [97]. Gas chromatography with flame-ionization detection [98] and gas chromatography with mass spectrometric detection [99] have also been used for the quantitation of DAG. The t-BDMS derivatives of different DAG molecular species exhibited major ions at *m/z* 313, 341, 653 and 654 which can be monitored for the quantitation of DAG.

Previous studies have shown that AA is effectively converted into a number of metabolites such as 12-HETE, 15-HETE and PGE_2 that can be separated by TLC or HPLC [100,101]. For TLC analysis of AA and its metabolites, the samples are extracted with chloroform–methanol–formic acid (20:80:1.25, v/v). The chloroform layer is separated and evaporated to dryness. The samples are spotted on a TLC plate

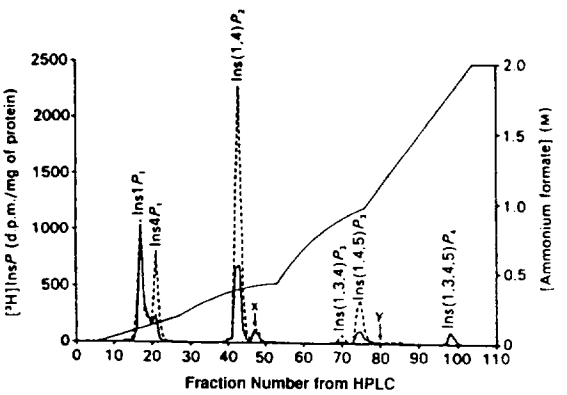

Fig. 12. HPLC of IPs from control (solid line) and carbachol stimulated (dotted line) smooth muscle samples using a non-linear gradient of ammonium formate. Muscle samples were incubated with [^{3}H]MI for 3 h and the not-incorporated [^{3}H]MI was removed by washing. The loaded samples were exposed to Ringer's buffer alone (for control) or the buffer containing carbachol (100 μM). The reaction was stopped with TCA and the TCA extract was washed with water-saturated diethyl ether. The washed extract was neutralized and injected onto the HPLC system equipped with a Partisil SAX anion-exchange column. IPs were separated by using gradients of ammonium formate buffer containing phosphoric acid (pH 3.7). Reprinted from Ref. [85] with permission.

and developed in acetate–hexane–acetic acid (30:50:1, v/v) and chloroform–methanol–acetic acid–water (80:8:1:0.8, v/v). As shown in Fig. 20, these solvent systems provide excellent separation of AA, 15-HETE, 12-HETE, 12-L-hydroxyheptadecatrienic acid and PGE_2 [102]. The HPLC analysis is performed by using a reversed-phase C_{18} column and a continuous gradient of water–acetic acid (100:0.05, v/v) and methanol–acetic acid (100:0.05, v/v) [18] or water–acetic acid (100:0.05, v/v) and acetonitrile [102]. The acetonitrile-based mobile phase provided good separation of AA and its metabolites (Fig. 21).

6. Quantitation of inositol phospholipids and inositol phosphates

The receptor-mediated changes in inositol phospholipids and IPs are commonly studied by radiolabelling PtdIns{4,5}P_2 with either [^{3}H]MI or [γ-^{32}P]ATP and measuring the radiolabelling of the individual IPs. For the radiolabelling

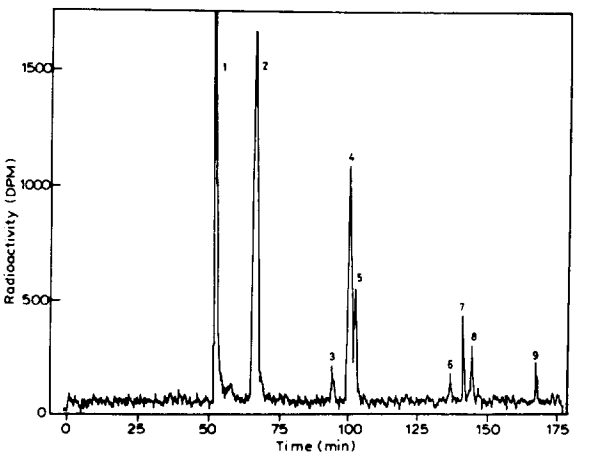

Fig. 13. HPLC separation of [^{3}H]MI-labelled IP isomers in cerebellar slices. Cerebellar slices were loaded with [^{3}H]MI and then homogenized in 10% TCA. The homogenate was centrifuged and the clear supernatant was neutralized with 0.1 M NaOH. The neutralized extract was injected onto a HPLC system equipped with a Partisil SAX column. IPs were eluted with stepwise gradients of ammonium phosphate from 0 to 1.75 M. IPs were detected with a radiometric detector. This method provided good separation of IP isomers, although the analysis time was almost 3 h. Reprinted from Ref. [88] with permission. 1 = I{1}P and I{3}P, 2 = I{4}P, 3 = I{1,3}P_2, 4 = I{1,4}P_2, 5 = I{3,4}P_2, 6 = glycerophosphoryl inositol kisphosphate, 7 = I{1,3,4}P_3, 8 = I{1,4,5}P_3, 9 = I{1,3,4,5}P_4.

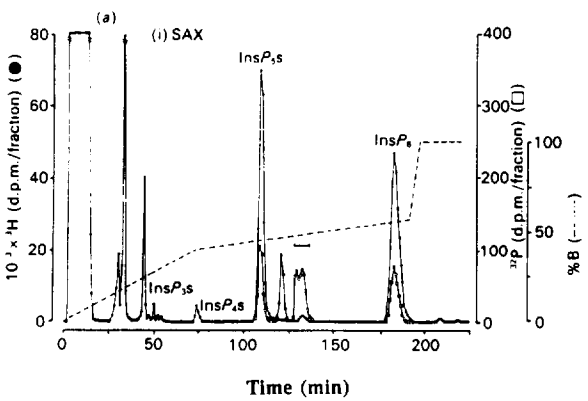

Fig. 14. Separation of IPs including IP_5 and IP_6 by a Partisphere SAX column with a 0–100% ammonium phosphate gradient. HL60 cells were loaded with [^{3}H]MI and then homogenized in 4 volumes of TCA. The TCA extract was neutralized and injected onto a gradient HPLC with a Partisphere SAX column and a radiometric detector. IPs were eluted with gradients of sodium phosphate and ammonium phosphate. Reprinted from Ref. [89] with permission.

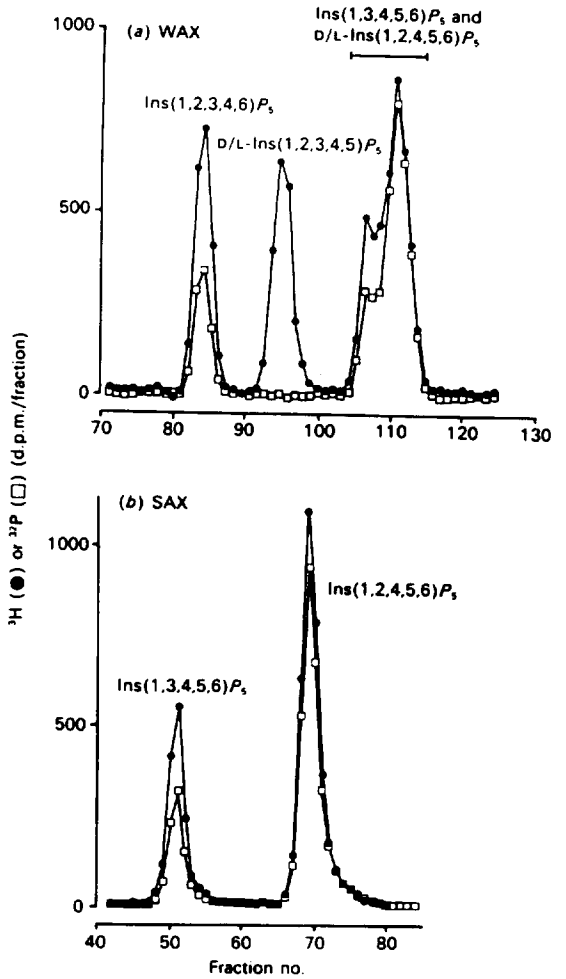

Fig. 15. Resolution of IP_5 isomers on a Partisphere WAX column using a 0–95% gradient of ammonium phosphate. NC4 cells were incubated with [^{32}P]P_i and then TCA extracts were prepared. The extracts were neutralized and chromatographed on a Partisphere WAX column. This method provided excellent separation of I{1,2,3,4,6}P_5 from I{1,2,3,4,5}P_5 but provided poor separation of I{1,2,4,5,6}P_5 and I{1,3,4,5,6}P_5 in tissue samples (upper chromatogram). The HPLC eluates containing I{1,2,4,5,6}P_5 and I{1,3,4,5,6}P_5 were collected and rechromatographed on a Partisphere SAX column that separated the two IP_5 isomers (lower chromatogram). Reprinted from Ref. [89] with permission.

method to accurately reflect changes in the compound's mass, it is essential that the tissue is labelled with a radioisotope to isotopic equilibrium [103,104]. This may be difficult because several studies have demonstrated that (1) the incorporation of [^{32}P]ATP or [^{3}H]MI into PtdIns{4,5}P_2 takes a couple of hours to a couple of days depending upon the type of the radioisotope and tissue samples [105–107], (2) the incorporation of ^{32}P into PtdIns{4,5}P_2 reached a maximum level within 2 h and then the labelling decreases [108]; however, the incorporation of ^{32}P into other phospholipids continued to increase after 2 h [108], (3) labelling tissues with [^{3}H]MI, requiring long incubation periods, may effect agonist response [109] and (4) the concentration of [^{3}H]MI at isotopic equilibrium differs in different tissues [109–113]. This also suggests that different phospholipids may require different incubation periods to reach isotopic equilibrium. The radiolabelling of phospholipids is also affected by the presence of endogenous unlabelled precursors such as MI or Ch in tissues [16]. It is therefore important to measure the time-course of radioisotope incorporation in individual phospholipid to ensure that all pools of the phospholipid have been labelled to isotopic equilibrium. This is particularly important when the radiolabelling method is being used for studying the metabolism of IPs. It is commonly believed that IP_3 and IP_4 are the sole metabolic precursors of IP_2 and IP and, thus, change in the levels of IP_3 and/or IP_4 causes proportional changes in the levels of IP_2 and IP [5]. However, recent studies have shown that IP_3 and IP_4 are exclusively released from PtdIns{4,5}P_2 while IP_2 and IP are released both from PtdIns{4}P and PtdIns{4,5}P_2 [22,23], thus suggesting that IP_3 may not be the sole source of IP_2 in tissues. Therefore, if both PtdIns{4}P and PtdIns{4,5}P_2 are not labelled to isotopic equilibrium, the radiolabelling method will provide inaccurate information regarding the metabolism of IPs in tissues.

To circumvent the disadvantages of the radiolabelling method, a previous study has used a radioreceptor assay for the mass analysis of I{1,4,5}P_3 and I{1,3,4,5}P_4 which may provide more accurate information than the radiolabelling method with regard to the mobilization of IPs [114]. The radioreceptor method, however,

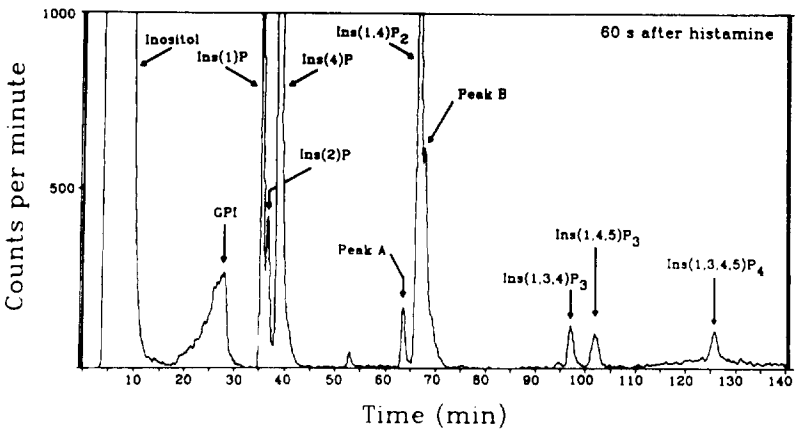

Fig. 16. HPLC separation of ^{3}H-labelled inositol phosphates in chromaffin cells. The cells were loaded with [^{3}H]MI and then homogenized with 20% TCA. The homogenate was neutralized and injected onto an HPLC system consisting of a Model 600E gradient controller (Waters), Partisil 10 SAX column and an on-line radioactivity detector. The IPs were eluted with a gradient of ammonium phosphate at pH 3.8. This method provided excellent separation of different IP isomers, although the analysis took more than 2 h. Reprinted from Ref. [91] with permission.

specifically measures I{1,4,5}P_3 or I{1,3,4,5}P_4; therefore, the metabolism of other phospholipids is not studied. Recently, several analytical procedures have been developed for the mass analysis of phospholipids and IPs. Most methods involve the extraction of lipids from tissue samples, chromatographic separation of individual lipids using TLC, cartridge-column or HPLC, and the detection of individual lipids by a suitable detector or chemical reaction. The aim of this section, therefore, is to discuss the methodologies for radiolabelling and mass analysis of IPs.

6.1. Radiolabelling

6.1.1. Radiolabelling with [^{3}H]myo-inositol

Cell culture, tissue homogenates or subcellular fractions (such as synaptosomes or microvessels) are incubated for 2 to 4 h at 37°C with [^{3}H]MI in Ringer's buffer (0.75 μM, 15 Ci/m mol). After incubation, the samples are centrifuged and the supernatant is discarded. The samples are then washed 4 times with Ringer's buffer. To study the receptor stimulation of PtdIns{4,5}P_2 hydrolysis, the loaded samples are incubated with the agonist and the radioactivity in different IPs is measured at different time intervals. Previous observation that carbachol increased the concentration but did not increase the radiolabelling of IP_2 and IP [22] and that IP_3 may not be the only source of IP_2 [23,115] complicates the interpretation of the radiolabelling data. Despite these disadvantages, radiolabelling with [^{3}H]MI has been extensively used for studying receptor-stimulated IP mobilization.

6.1.2. Radiolabelling with [γ-^{32}P]ATP

Several studies have shown that incubation of tissues with [γ-^{32}P]ATP leads to the incorporation of ^{32}P into phospholipids because of the presence of the enzyme lipid kinase [116,117]. Tissues can be labelled with ^{32}P either by in vitro incubation of tissues with [γ-^{32}P]ATP or by intracerebral injection of [γ-^{32}P]ATP [108,118]. Previous observations that (1) the labelling of PtdIns[4}P or PtdIns[4,5}P_2 reached a maximum level within 2 h after injection and, with prolonged time, the labelling declined and (2) the labelling of other phospholipids continued to rise after 2 h [108] suggest differences in the time taken by different phospholipids to reach isotopic equilibrium.

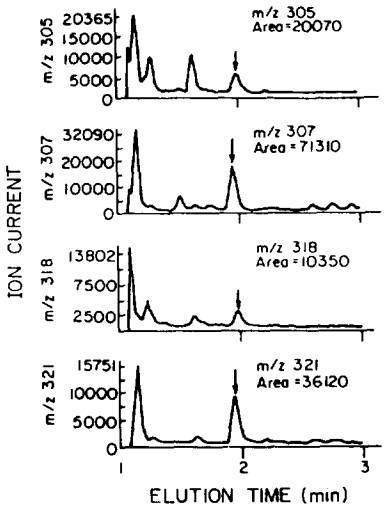

Fig. 17. Gas chromatographic–mass spectrometric analysis of natural and d_6-MI obtained from the dephosphorylation of IP_4 in canine myocytes. The extraction and chromatographic separation of IPs are described in Fig. 11. The fractions containing individual IPs were pooled and lyophilized. Each IP was dephosphorylated by alkaline phosphatase and then desalted by adding a stoichiometric amount of solid $Ba(OH)_2$. The desalted samples were again lyophilized and the dried residue was derivatized with a mixture of BSTFA and TMCS. The derivatized samples were analyzed by a gas chromatograph–electron-impact (EI) mass spectrometer (GC–MS). The GC–MS was programmed in the SIM mode to monitor ions at *m/z* 305, 318, 307 and 321. Ions at *m/z* 305 and 318 are the major ions produced by the natural MI and ions at *m/z* 307 and 321 are the major ions produced by the internal standard (d_6-MI). Reprinted from Ref. [93] with permission.

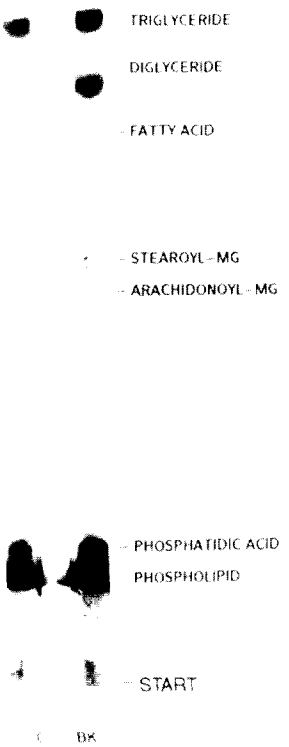

Fig. 18. TLC separation of lipids from neuronal culture. Neuronal cells, loaded with [^{3}H]AA, [^{3}H]stearic acid and [^{3}H]palmitic acid, were extracted with 3.6 ml of cold chloroform–methanol (2:1, v/v) and then with 2.4 ml of chloroform–2 *M* KCl (1:1, v/v). The organic layer was collected and spotted on the TLC plate. The plate was developed in ethyl acetate–iso-octane–acetic acid–water (93:47:21:100, v/v). The lipid moieties were detected by autoradiography. C: control and BK: bradykinin treated. Reprinted from Ref. [97] with permission.

6.2. Mass analysis of inositol phospholipids

6.2.1. Analysis of inorganic phosphate

The measurement of inorganic phosphate (P_i) provides a simple and sensitive measurement of IP mass in tissues. The chromatographic effluents containing the individual phospholipids are freed of phosphate by using Dowex 1 X-8. The samples are then treated with alkaline phosphatase and the concentration of P_i is measured by using the molybdate reagent containing citrate [119]. It has been suggested that citrate prevents the hydrolysis of ATP, a natural precursor of P_i that gives false P_i values [119]. The color reaction is monitored at 660 nm. An important disadvantage of the method is the interference by IP_6 with the analysis of P_i [120]. The interference can be eliminated by extracting the sample after molybdenum reaction with isobutanol–*n*-heptane (3:2, v/v) and measuring the optical density of the organic layer at 725 nm [120]. A previous study has described an on-line post-column detection of P_i that provides direct quantitation of phospholipids in tissue extracts [90]. Hanaoka et al. [121] have described an HPLC method using conductivity detection for the quantitation of phosphate and other anions; however, the possible application of this method

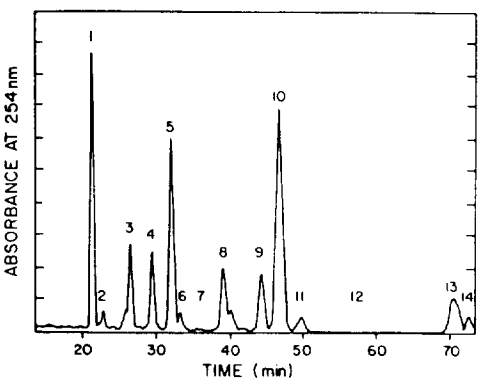

Fig. 19. HPLC separation of DAG standards. DAG standards (1 = 18:2–18:2, 3 = 16:0–20:4, 4 = 15:0–15:0, 5 = 16:0–18:2, 6 = 14:0–16:0, 8 = 18:0–20:4, 9 = 18:1–18:1, 10 = 16:0–18:1, 11 = 16:0–16:0, 13 = 18:0–18:1, 14 = 16:0–18:0) or extracted samples were incubated with 0.5 ml of dry pyridine containing 25 mg of 3,5-dinitrobenzoyl chloride for 20 min at 60°C. After that, the samples were mixed with 2 ml of 0.1 *M* HCl and extracted 3 times with *n*-hexane. The extracts were combined and evaporated to dryness under nitrogen. The dried extract was redissolved in 150 μl of acetonitrile and injected onto an HPLC containing a C_{18} reversed-phase column. The column eluates were dried and the radioactivity was determined by scintillation counting. Reprinted from Ref. [97] with permission.

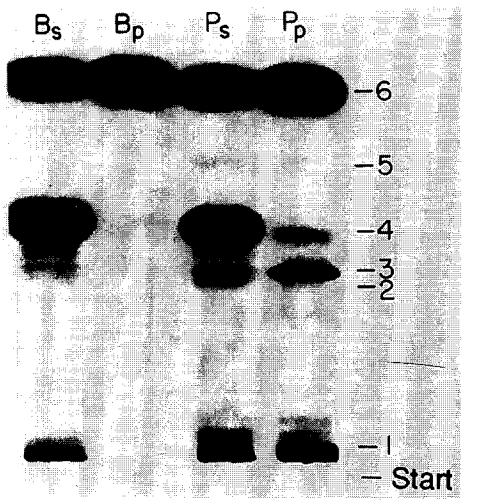

Fig. 20. Chromatographic separation of AA and its metabolites by TLC. Samples from fetal rabbit brain (B) or placenta (P) was homogenized in 10 volumes of phosphate buffer (pH 7.0, 50 m*M*) containing 1 m*M* EDTA and 0.1% gelatin. The homogenate was centrifuged at 100 000 *g* for 1 h. The pellets and supernatant were separated. The pellet was reconstituted to the original volume with homogenization buffer. Aliquots of whole homogenate, supernatant or the pellet were incubated with [^{14}C]AA and 2 m*M* $CaCl_2$ for 15–30 min. The reaction was stopped by adding 1 ml of methanol–chloroform–formic acid (80:20:1.25, v/v). The mixture was centrifuged and the sample was reextracted with a similar mixture. The two extracts were pooled and mixed with chloroform and water to achieve a mixture of chloroform–methanol–water (8:4:3, v/v). The chloroform layer was collected and evaporated to dryness. The dried residue was redissolved in chloroform and applied on a TLC plate. The plate was developed in ethyl acetate–hexane–acetic acid (30:50:1, v/v) and the different spots were visualized by autoradiography. 1 = PGE_2, 3 = 12-HETE and 6 = AA. Reprinted from Ref. [102] with permission.

to the quantitation of phospholipids has not yet been described.

6.2.2. UV absorbance

As discussed earlier, phospholipids exhibit UV absorbance in the 200-nm range [22] that has been used in several HPLC methods for the quantitative analysis of phospholipids [62,63] and phospholipid fatty acids [122]. However, the UV absorbance method lacks sensitivity and can not be used for the trace analysis of phospholipids. The sensitivity of the UV absorption method is increased for inositol phospholipids by derivatizing PtdIns{4}P and PtdIns{4,5}P_2 with 9-anthryldiazomethane and measuring the absorbance of the derivative at 254 nm [56].

6.2.3. Mass spectrometry

HPLC coupled to a mass spectrometer (HPLC–MS) through a thermospray interface provides an important tool for the monitoring of HPLC effluents [123]. Kim and Salem [58] have developed simple HPLC–MS methods for the detection of phospholipids separated by HPLC. The detection of phospholipids by MS is highly specific, thus circumventing the disadvantages of the detection of underivatized lipids by UV detection. Quantitation of phospholipids can be achieved by programming the MS in the selective-ion monitoring (SIM) mode. However, more research is needed to use HPLC–MS for the quantitation of lipids in tissue extracts.

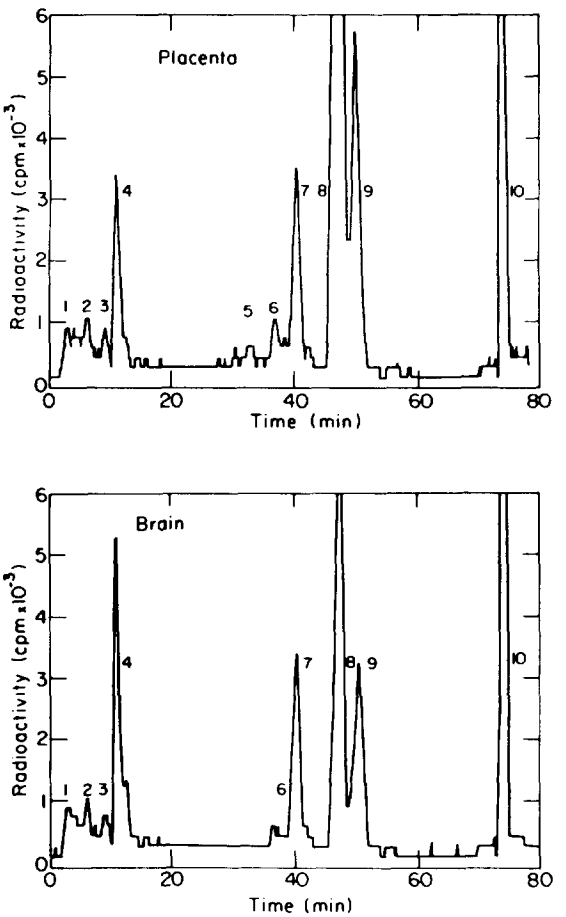

Fig. 21. HPLC profile of [^{14}C]AA and its metabolites in placenta and brain. The final chloroform extract, obtained as described in Fig. 20, was analyzed on a C_{18} column (ODS, 5μm). The mobile phase was acetonitrile in 0.1% acetic acid. Radioactivity was determined by the radioactive flow detector. Peak identification: 4 = PGE_2, 7 = HHT, 8 = 15-HETE, 9 = 12-HETE and 10 = AA. Reprinted from Ref. [102] with permission.

6.3. Mass analysis of inositol phosphates

6.3.1. Enzymatic analysis

This assay is based on the oxidation of MI to scyllo-inosose and the reduction of NAD^+ to NADH by MI dehydrogenase (MIDH). Since the accumulation of NADH inhibits MIDH activity, the reaction is coupled with a scavenge reaction, e.g. the malate dehydrogenase (MDH) reaction which reduces oxaloacetate to malate [124], the alcohol dehydrogenase reaction [125], the diphorase reaction with resazurin as the substrate [22,126] or a bioluminescence reaction [85]. The quantitation of IPs by the enzymatic method is a multi-step process involving the extraction of tissues with TCA or PCA, removal of endogenous MI by passing the extract through a Dowex-50W column, separation of IP, IP_2, IP_3 and IP_4 by anion-exchange chromatography or HPLC, dephosphorylation of each IP to MI by alkaline phosphatase and then measuring the concentration of MI in each fraction. The enzymatic assay has been recently adapted for Sep-Pak eluates where MI is measured by using diphorase and resazurin [22] and for HPLC eluates where MI is measured with a bioluminescence assay [85]. These methods are sensitive and require only a small sample size. However, there are some limitations of the enzymatic methods, e.g. (1) the rate of dephosphorylation of IP_4 is slower than that of IP, IP_2 and IP_3, (2) IP_5 and IP_6 are very poorly dephosphorylated and (3) the presence of salt interferes with dephosphorylation of IPs.

6.3.2. Metal–dye detection

A previous study has shown that yttrium, which is a tervalent transition-metal ion, binds with high affinity both to cation-specific dyes such as PAR and to polyanions such as IPs [82,89]. The formation of a metal–dye complex causes a decrease and the formation of an anion–metal complex causes an increase in the absorbance [82]. This metal–dye reaction has been successfully used for the quantitation of pmol amounts of inositol phosphates by HPLC coupled with a post-column reaction chamber and a UV detection [82,89]. The absorbance of the metal–dye complex is measured at 546 nm or 520 nm. The HPLC technique described previously separates IP_2 and higher poly-IP isomers from each other and from interfering phosphorylated substances and the IPs are quantitated by the metal–dye reaction. IP and IP_2 are not detected by this method because GTP, glucose 1,6-bisphosphate or sedoheptulose 1,7-bisphosphate interfere with the chromatographic separation of IP_2 and phosphoenolpyruvate; phosphoglycerate

and 6-phosphogluconate may interfere with the chromatography of IP [82]. This method, however, requires a complex and expensive HPLC system and extensive knowledge of HPLC separation techniques.

6.3.3. Nuclear magnetic resonance

High-resolution NMR has been recently described to be an important tool for the identification of IP isomers. Although ^{1}H-NMR and ^{13}C-NMR both have been used for this purpose, the ^{1}H-NMR spectrum is more sensitive and rapid than ^{13}C-NMR spectra [128]. For NMR analysis it is important to isolate the compound of interest from the sample matrix and to determine its concentration because approximately 0.1–0.15 μmol of the compound may be needed to accumulate satisfactory data [128]. Therefore, the samples are separated by HPLC and the fractions containing the individual IPs are pooled and further purified by ion-exchange column chromatography. The compounds are freeze-dried and the dried residue is dissolved in 1 ml of D_2O (isotopic purity >99%). The sample is treated with Chelex 100 resin and the purified sample (pH 6 or 9) is further freeze-dried [89,128,129]. The dried samples are again dissolved in D_2O and transferred into NMR tubes and dried. The sample is then subjected to NMR analysis for either one-dimensional ^{1}H-NMR or ^{13}C-NMR spectra [89,127]. Fig. 22 shows the ^{1}H-NMR spectra of two different pools of samples containing IP_3 isomers [129]. Fig. 22a,b represents I{1,3,4}P_3 and I{1,5,6}P_3, respectively. The NMR method, however, may not be suitable for the routine analysis of IPs because it requires expensive equipment and extensive knowledge of NMR technology.

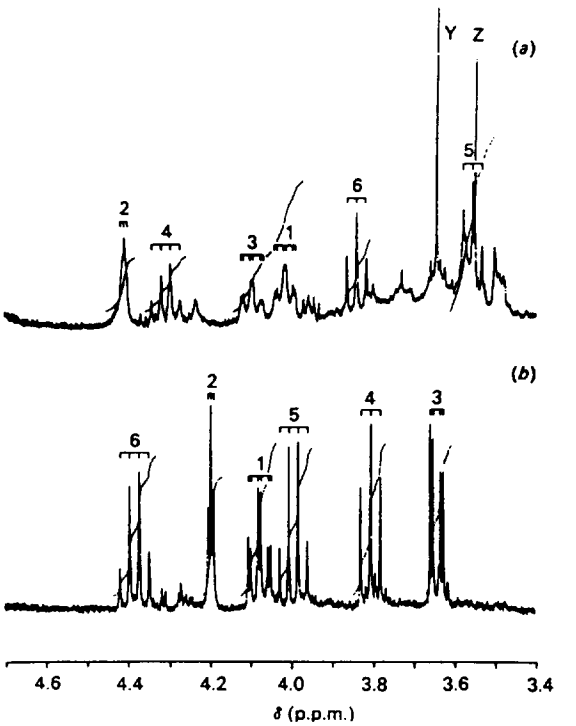

Fig. 22. Proton-NMR profile of I{1,3,4}P_3 (a) and I{1,5,6}P_3 (b) isolated from turkey erythrocytes. Turkey blood (3 l) was mixed with 1 *M* PCA (1:1, v/v) and the mixture was centrifuged. The pH of the PCA extract was adjusted to 5 with KOH. The clear supernatant was lyophilized. The dried extract was dissolved in 700 ml of water and then subjected to charcoal treatment for the removal of nucleotides. The resulting extract was again lyophilized, the dried residue was redissolved in 200 ml of water and the pH was adjusted to 8. The solution was applied to a Q-Sepharose formate column and the IPs were eluted with a 0–1.2 *M* gradient of ammonium formate. The fractions containing individual IPs were pooled and lyophilized. The fractions were then analyzed by a HPLC equipped with Mono Q column. IP isomers were eluted with stepwise gradient of YCl_3. The fractions containing the different isomers of IP_3 were subjected to NMR analysis. Resonances are marked according to the MI ring. Reprinted from Ref. [129] with permission.

6.3.4. Gas chromatography with flame ionization detection or mass spectrometry

Several investigators have used an FID detector for the quantitation of I{1}P in tissue samples [92]. Although the method is simple and sensitive, it does not detect other IPs and, thus, its application is limited. DaTorre et al. [93] and Turk et al. [130] have developed a GC–MS method that measures TMD derivatized MI by electron-impact and chemical-ionization MS, respectively. For the analysis of IPs, it is important to separate the different IPs and dephosphorylate each IP into MI and measure the concentration of MI [93]. For GC–MS analysis tissue samples are mixed with d_6-MI as an internal standard and extracted with TCA. The acid is removed by washing the sample with

water-saturated diethyl ether and the aqueous phase is lyophilized. The dried sample is redissolved in Tris buffer (pH 8.8, 0.01 *M*) and poured onto an anion-exchange resin. IP, IP_2, IP_3 and IP_4 are separated by using gradients of ammonium sulfate. Each IP is dephosphorylated with alkaline phosphatase and MI is derivatized with a reagent containing 25 μl each of pyridine and BSTFA + 0.1% TMCS. The derivatized sample is analyzed by a GC–EI-MS [99] or GC–CI-MS [129]. A previous study has shown that the major EI ions produced by the natural MI are at *m/z* 305 and 318 and the major ions produced by d_6-MI are at *m/z* 307 and 321 [93]. Quantitation is performed by determining the peak-area ratio for MI and d_6-MI as described previously. For more sensitivity the GC–MS is programmed to monitor only selected ions (selected-ion monitoring mode), e.g. at *m/z* 305, 307, 318 and 321, and determining the area under the curve for each peak.

7. Conclusions

The mobilization of inositol phospholipids, IPs and related compounds is usually studied with the radiolabelling method in which the different phospholipid pools are labelled with ^{32}P or [^{3}H]MI before stimulation. The loaded samples are extracted and the individual compounds are separated by TLC, HPLC or cartridge-column chromatography. The choice of chromatographic method will depend upon the objectives of the experiments. TLC and cartridge-column chromatography may not provide effective separation of the individual IP isomers but provide rapid separation of phospholipid or inositol phosphate classes. However, the HPLC methods may provide good separation of the IP isomers. After chromatographic separation, the incorporation of radioactivity in each molecule is measured by scintillation counting. However, for the radiolabelling method to accurately reflect changes in the compound's mass, it is essential that the different phospholipid pools are labelled to isotopic equilibrium, which is difficult to achieve. Recently, several mass-analysis methods have been developed that circumvent the disadvantages of the radiolabelling method. However, most mass-analysis methods are complex and time-consuming.

References

[1] M. Shinitzky, in M. Shinitzky (Editor), *Physiology of Membrane Fluidity*, Vol. 1, CRC Press, Boca Raton, FL, 1984, p. 1.
[2] K.W.A. Wirtz, in P.C. Jost and O.H. Gruffith (Editors) *Lipid–Protein Interactions*, Vol. 1, Wiley, New York, NY, 1982, p. 151.
[3] J.R. Dave and R.J. Witorsch, *Biochim. Biophys. Acta*, 772 (1984) 321.
[4] R.S. Rana and L.E. Hokin, *Physiol. Rev.*, 70 (1990) 115.
[5] B.W. Agranoff, F. Eisenberg, G. Hauser, N.S. Hawthorne and R.H. Michell, in J.E. Blersedale, J. Eichberg and G. Hauser (Editors), *Inositol and Phosphoinositides*, Humana Press, Clifton, NJ, 1985, p. xxi.
[6] D.L. Lips, P.W. Majerus, F.R. Gorga, A.T. Young and T.L. Benjamin, *J. Biol. Chem.*, 264 (1989) 8759.
[7] L.A. Serunian, M.T. Habe, T. Fukui, J.W. Kim, S.G. Rhee, J.M. Lowenstein and L.C. Cantley, *J. Biol. Chem.*, 264 (1989) 17809.
[8] M.J. Berridge and R.F. Irvine, *Nature*, 341 (1988) 107.
[9] R.H. Michell, *Biochim. Biophys. Acta*, 414 (1975) 81.
[10] A.K. Singh, *NeuroToxicology*, 14 (1993) 417.
[11] R.F. Irvine, *Biochem. Soc. Trans.*, 17 (1989) 6.
[12] B.B. Biswas, S. Biswas, S. Chakrabarti and B.P. De, in W.W. Wells and F. Eisenberg (Editors), *Cyclitols and Phosphoinositides*, Academic Press, New York, NY, 1978, p. 57.
[13] E.A. Dennis, *The Enzymes*, 16 (1983) 307.
[14] R.W. Bonser, N.T.T. Thompson and L.G. Garlard, in E. Reid, G.M.W. Cook and J.P. Luzid (Editors), *Cell Signalling Experimental Strategies*, Royal Society of Chemistry, Cambridge, UK, 1990, p. 59.
[15] Y.A. Hannun and R.M. Bell, *Science*, 243 (1989) 500.
[16] N.M. Dean and M. Beaven, *Anal. Biochem.*, 183 (1989) 199.
[17] J. Folch, M. Lee and S.M. Stanley, *J. Biol. Chem.*, 226 (1957) 497.
[18] S.C. Amruthesh, J.R. Falk and E.F. Ellis, *J. Neurochem.*, 58 (1992) 503.
[19] E. Golding, S. Harel, A. Tomer and E. Yavin, *J. Neurochem.*, 48 (1987) 695.
[20] T. Radenberg, P. Scholz, G. Bergmann and G.W. Mayr, *Biochem. J.*, 264 (1989) 323.
[21] G.W. Mayr and W. Dietrich, *FEBS Lett.*, 254 (1988) 585.
[22] A.K. Singh, *J. Chromatogr.*, 581 (1992) 1.
[23] M.J. Brammer and K. Weaver, *J. Neurochem.*, 53 (1989) 399.

[24] H. Ishii, T.M. Connolly, T.E. Bross and P.W. Majerus, *Proc. Natl. Acad. Sci. USA*, 83 (1986) 3051.
[25] D.F. Dixon and L.E. Hoken, *J. Biol. Chem.*, 260 (1988) 16068.
[26] P.T. Hawkins, C.L. Berrie, A.J. Morris and C.P. Dowens, *Biochem. J.*, 243 (1987) 211.
[27] R. Wilson and J.R. Sargent, *J. Neurochem.*, 61 (1993) 290.
[28] G. Rouser, G. Kritchevsky, A. Yamamoto, G. Simon, C. Gallic and A.J. Bauman, *Methods Enzymol.*, 14 (1969) 272.
[29] P.T. Hawkins, R.H. Michell and C.G. Kirk, *Biochem. J.*, 218 (1984) 785.
[30] G. Rouser, M. Lees and G.H. Sloane-Stanley, *J. Biol Chem.*, 226 (1957) 497.
[31] N.G. Clarke and R.M.C. Dawson, *Biochem. J.*, 195 (1981) 301.
[32] G. Rouser, G. Kritchevsky and A. Yamamoto, in G.V. Marinetti (Editor), *Lipid chromatographic Analysis*, Vol. 1, Dekker, New York, NY, 1967, p. 99.
[33] J.M. Stein, G. Smith and J.P. Luzio, in R.F. Irvine (Editor), *Methods in Inositide Research*, Raven Press, New York, NY, 1990, p. 157.
[34] J.R. Broich, S. Goldner, G. Groudet, S. Andryauskas, C.J. Vmber and D.B. Hoffman, *J. Chromatogr.*, 80 (1973) 275.
[35] P.C. Mati, *J. Forensic Sci.*, 16 (1971) 245.
[36] R.J. Bussey and R.E. Backer, *Clin. Chem.*, 20 (1974) 302.
[37] N.C. Jain, W.J. Leung, R.D. Budd and T.C. Snoath, *J. Chromatogr.*, 115 (1975) 519.
[38] P. Owen, A. Pendleburg and A.C. Moffat, *J. Chromatogr.*, 161 (1978) 187.
[39] F. Vitiello and J.P. Zanetta, *J. Chromatogr.*, 166 (1978) 637.
[40] J.G. Parsons and S. Patton, *J. Lipid Res.*, 8 (1967) 696.
[41] A.N. Siakotos and G. Rouser, *J. Am. Oil Chemists' Soc.*, 42 (1965) 913.
[42] G. Rouser, G. Kritchevsky, G. Simon and G. Nelson, *Lipids*, 2 (1967) 37.
[43] G. Rouser, G. Kritchevsky, A.N. Siakotos and A. Yamamoto, in C.D. Tedeschi (Editor), *An Introduction to Neuropathology: Method and Diognosis*, Little Brown, New York, NY, 1969.
[44] M. Söderberg, C. Edlund, C.I. Alafuzoff, K. Kristensson and G. Dallner, *J. Neurochem.*, 59 (1992) 1646.
[45] J. Schacht, *J. Lipid Res.*, 19 (1978) 1063.
[46] F.B.St.C. Palmer, *J. Lipid Res.*, 22 (1981) 1296.
[47] A. Schibeci and J. Schacht, *Biochem. Pharmacol.*, 26 (1977) 1769.
[48] A. Orsulakova, E. Stockhorst and J. Schacht, *J. Neurochem.*, 26 (1976) 285.
[49] E. Stockhorst and J. Schacht, *Acta Otolaryng.*, 83 (1977) 401.
[50] J. Schacht, *J. Neurochem.*, 27 (1976) 1119.
[51] S. Lodhi, N.D. Weiner and J. Schacht, *Biochim. Biophys. Acta*, 426 (1976) 781.
[52] L.L. Dugan, P. Demediuk, C.E. Pendley II and L.A. Horrocks, *J. Chromatogr.*, 378 (1986) 317.z
[53] W.S.M. Geurts van Kessel, W.M.A. Hax, R.A. Demel and J. de Gier, *Biochim. Biophys. Acta*, 486 (1977) 524.
[54] M.F. Caboni, G. Lercker and A.M. Ghe, *J. Chromatogr.*, 315 (1984) 223.
[55] B. Rivnag, *J. Chromatogr.*, 294 (1984) 303.
[56] T. Nakamura, Y. Hatori, K. Yamada, M. Ikeda and T. Yuzuriha, *Anal. Biochem.*, 179 (1989) 127.
[57] K. Yamada, S. Abe, K. Katayama and T. Sato, *J. Chromatogr.*, 424 (1988) 367.
[58] H.Y. Kim and N. Salem, Jr., *Anal. Chem.*, 59 (1987) 722.
[59] H.Y. Kim and N. Salem, Jr., *Anal. Chem.*, 58 (1986) 9.
[60] P.W. Majerus, T.S. Ross, T.W. Cunningham, K.K. Caldwell, A.B. Jefferson and V.S. Barsal, *Cell*, 63 (1990) 459.
[61] K.R. Auger, L.A. Serumian and L.C. Cartleg, in R.F. Irvine (Editor), *Methods in Inositide Research*, Raven Press, New York, NY, 1990, p. 159.
[62] M. Hokin-Neaverson and K. Sadeghian, *J. Chromatogr.*, 120 (1976) 502.
[63] M.E. Koch-Kallnbach and H. Diring, *Z. Hoppe-Seyler, Physiol. Chem.*, 358 (1977) 367.
[64] A. Emilsson and R. Sundler, *J. Biol. Chem.*, 259 (1984) 3111.
[65] A. Desjobert and F. Petek, *Bull. Soc. Chem. Biol.*, 38 (1956) 871.
[66] C. Grado and C.E. Ballou, *J. Biol. Chem.*, 236 (1961) 54.
[67] D.M. Brown and J.C. Steward, *Biochim. Biophys. Acta*, 125 (1966) 413.
[68] U.B. Seiffert and B.W. Agranoff, *Biochim. Biophys. Acta*, 98 (1965) 574.
[69] M.J. Berridge, R.M. Dawson, C.P. Downes, J.P. Heslop and R.F. Irvine, *Biochem. J.*, 212 (1983) 473.
[70] R.B. Ellis, T. Galliard and J.N. Hawthorne, *Biochem. J.*, 88 (1963) 125.
[71] G.P. Heathers, J. Twyla, L.J. Rubin, P.B. Corr and A.S. Evers, *Anal. Chem.*, 176 (1989) 109.
[72] G.R. Bartlett, *Anal. Biochem.*, 124 (1982) 425.
[73] P.A. Lanzetta, L.J. Alvarez, P.S. Reinach and O.A. Candia, *Anal. Biochem.*, 100 (1979) 95.
[74] S. Christensen and H. Harbak, *J. Chromatogr.*, 533 (1990) 201.
[75] R.F. Irvine, A.J. Letcher, D.J. Lander and C.P. Downes, *Biochem. J.*, 223 (1984) 237.
[76] R.F. Irvine, E.E. Anggard, A.J. Letcher and C.P. Downes, *Biochem. J.*, 229 (1985) 505.
[77] I.R. Batty, S.R. Nahooski and R.F. Irvine, *Biochem. J.*, 232 (1985) 211.
[78] J.P. Heslop, R.F. Irvine, A.H. Tashjian and M.J. Berridge, *J. Exp. Biol.*, 119 (1985) 395.
[79] L.J. Reed and J. deBellerache, *J. Neurochem.*, 50 (1988) 1566.

[80] W.R. Mathews, D.M. Guido and R.M. Huff, *Anal. Biochem.*, 168 (1988) 63.
[81] M. Brammer and K. Weaver, *J. Neurochem.*, 53 (1989) 407.
[82] G.W. Mayr, *Biochem. J.*, 254 (1988) 585.
[83] D.C. Johnson and W.R. LaCourse, *Anal. Chem.*, 62 (1990) 589.
[84] R.E. Smith, R.A. MacQuarrie and R.S. Jope, *J. Chromatogr. Sci.*, 27 (1989) 491.
[85] S.A. Prestwich and T.B. Bolton, *Biochem. J.*, 274 (1991) 663.
[86] J.L. Meek and F. Nicoletti, *J. Chromatogr.*, 351 (1986) 303.
[87] R.J. Barnaby, *Anal. Biochem.*, 199 (1951) 75..
[88] P.S. Sastry, J.F. Dixon and L.E. Hokin, *J. Neurochem.*, 58 (1992) 1079.
[89] L.R. Stephens, P.T. Hawkins, A.F. Stanley, T. Moore, D.R. Poyner, P.J. Mirrus, M.R. Hanley, R.R. Kay and R.F. Irvine, *Biochem. J.*, 275 (1991) 485.
[90] J.L. Meek, *Proc. Natl. Acad. Sci. USA*, 83 (1986) 4162.
[91] K.A. Stauderman and R.M. Pruss, *J. Neurochem.*, 54 (1990) 946.
[92] M.R. Hirvonen, H. Lihtamo and K. Savolainen, *Neurochem Res.*, 13 (1988) 957.
[93] S.D. DaTorre, P.B. Corr and M.H. Creer, *J. Lipid Res.*, 31 (1980) 1925.
[94] S.E. Rittenhouse and J.P. Sesson, *J. Biol. Chem.*, 260 (1985) 8657.
[95] D.A. Kennerly, C.W. Parker and T.J. Sullivan, *Anal. Biochem.*, 98 (1979) 123.
[96] J. Preiss, C.R. Loomis, W.R. Bishop, R. Stein, J.E. Niedel and R.M. Bell, *J. Biol. Chem.*, 261 (1986) 8597.
[97] A.C. Allen, C.M. Gammon, A.H. Ousley, K.D. McCarthy and P. Morell, *J. Neurochem.*, 58 (1992) 1130.
[98] K. Itoh, A. Suzuki, Y. Kuroki and T. Akino, *Lipids*, 20 (1985) 611.
[99] J.J. Myher, A. Kuksis, L. Marai and S.K.F. Yeung, *Anal. Chem.*, 50 (1978) 557.
[100] J.R. Falck, V.J. Schueler, H.R. Jacobson, A.K. Siddanta, B. Pramanic and J. Capdevila, *J. Lipid Res.*, 28 (1987) 840.
[101] M.P. Junier, F. Drag, I. Blair, J. Capdevila, E. Dishman, J.R. Falck and S.R. Ojeda, *Endocrinology*, 126 (1990) 1534.
[102] E. Goldin, S. Harle, A. Tomer and E. Yavin, *J. Neurochem.*, 48 (1987) 695.
[103] G.P. Bradford and R.P. Rubin, *J. Biol. Chem.*, 261 (1986) 15644.
[104] D. Portilla and A.R. Morrison, *Biochim. Biophys. Res. Commun.*, 14 (1986) 644.
[105] E.B. Stubbs, Jr., J.A. Kelleher and G.Y. Sun, *Biochim. Biophys. Acta*, 958 (1988) 247.
[106] R. Chandrasekhar, H.M. Huang, G.Y. Sun, *J. Pharmacol. Exp. Ther.*, 245 (1988)120.
[107] D.A. Horstman, H. Takemura and J.W. Putney Jr., *J. Biol. Chem.*, 263 (1988) 15297.
[108] G.Y. Sun and T.N. Lin, *Life Sci.*, 44 (1989) 889.
[109] D.A. Horstman, H. Takemura and J.W. Putney Jr., *J. Biol. Chem.*, 263 (1988) 15297.
[110] A.P. Tarver, W.G. King and S.E. Rittenhouse, *J. Biol. Chem.*, 262 (1987) 17268.
[111] D. Portilla and A.R. Morrison, *Biochim. Biophys. Res. Commun.*, 140 (1986) 644.
[112] P.G. Bradford and R.P. Rubin, *J. Biol. Chem.*, 261 (1986) 15644.
[113] K. Koreh and M.E. Monoqo, *J. Biol. Chem.*, 261 (1986) 88.
[114] R.A.J. Challiss, I.H. Battly and S.R. Nahorski, *Biochim. Biophys. Res. Commun.*, 157 (1988) 684.
[115] J.G. Baird and S.R. Nahorski, *Biochem. J.*, 267 (1990) 835.
[116] M.R. Hokins and L.E. Hokins, *Biochim. Biophys. Acta*, 84 (1964) 563.
[117] R.P. Schneider and L.B. Kirschner, *Biochim. Biophys. Acta*, 202 (1970) 283.
[118] C.E. King, L.R. Stephens, P.T. Hawkins, G.R. Guy and R.H. Michell, *Biochem. J.*, 244 (1987) 209.
[119] P.A. Lanzetta, L.J. Alvarez, P.S. Reinach and O.A. Candia, *Anal. Biochem.*, 100 (1979) 95.
[120] G.C.J. Irving and D.J. Cosgrove, *Anal. Biochem.*, 36 (1970) 381.
[121] Y. Hanaoka, T. Murayama, S. Muramoto, T. Matsuura and N. Nanpa, *J. Chromatogr.*, 239 (1982) 537.
[122] Y. Nakagawa and K. Waku, *J. Chromatogr.*, 381 (1986) 225.
[123] C.R. Blakely and M.L. Vestal, *Anal. Chem.*, 55 (1983) 750.
[124] L.C. MacGregor and F.M. Matschinsky, *Anal. Biochem.*, 141 (1984) 382.
[125] J.A. Shayman, A.R. Morrison and O.H. Lowry, *Anal. Biochem.*, 162 (1987) 582.
[126] J.A. Maslanski and W.B. Busa, in R.F. Irvine (Editor), *Methods in Inositide Research*, Raven Press, New York, NY, 1990, p. 113.
[127] G.W. Mayr, in R.F. Irvine (Editor), *Methods in Inositide Research*, Raven Press, New York, NY, 1990, p. 83.
[128] P. Scholz, G. Bergmann and G.W. Mayr, in R.F. Irvine (Editor), *Methods in Inositide Research*, Raven Press, New York, NY, 1990, p. 65.
[129] T. Redenberg, P. Scholz, G. Bergmann and W. Mayr, *Biochem. J.*, 264 (1989) 323.
[130] J. Turk, B.A. Wolf and M.L. McDaniel, *Biomed. Environ. Mass Spectrom.*, 13 (1986) 237.