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SEPARATION OF MAJOR PHOSPHOLIPID CLASSES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND SUBSEQUENT ANALYSIS OF PHOSPHOLIPID-BOUND FATTY ACIDS USING GAS CHROMATOGRAPHY

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SUMMARY

A sensitive high-performance liquid chromatographic method for the separation of major phospholipid (PL) classes in biological materials is described. Using this method it was easy to separate P-cholin, P-ethanolamine, P-serine, P-inositol, cardiolipin, sphingomyelin, lyso-P-choline and lyso-P-ethanolamine from skeletal and cardiac muscle samples. The method is based on the simultaneous use of a pH gradient and a polarity gradient. This procedure can easily be modified to optimize the separation of PLs from very different tissues. Subsequent analysis of the PL-bound fatty acids (FAs) by gas chromatography resulted in a well separated FA pattern. Following this FA separation it was possible to recalculate the specific PL content in the original sample.

INTRODUCTION

Phospholipids (PLs) are components of biological membranes with various compositions of the polar head groups and the ester-linked fatty acids (FAs). The physiological properties of membranes, such as consistency, thickness, fluidity and associated functions, are influenced by these structural differences[1]. For example, in a normal mammalian muscle cell the following major PL classes are present in various amounts[2]: P-choline (PC), P-ethanolamine (PE), P-serine (PS), P-inositol (PI), cardiolipin (C), plasmalogen PC and plasmalogen PE. However, the isolation and quantitation of the PL content in complex samples such as muscle is difficult, especially since the PL exist in very small amounts.

Several methods for the separation of PL from a variety of tissues have been reported. Most procedures employ thin-layer chromatography (TLC)[3]. A common stationary phase for TLC separation is silica gel or alumina[4]. However, modified materials such as diethylaminoethylsilica gel have also been used successfully[5]. Most TLC methods employing mobile phases with mixtures of chloroform–methanol–water are often modified with small amounts of acetic acid, ammonia solution and other

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solvents[6]. Detection of PL spots involves oxidation with sulphuric acid or staining with reagents such as molybdenum blue for choline-containing PL[6]. Subsequent determination of the PL content requires methods such as densitometry[4] or colorimetric measurement of PL phosphorus[7]. Qualitative analysis of TLC procedures in PL studies is simple, cheap and gives good results, but detailed quantification is extremely time consuming and requires extensive technical equipment.

To a more limited extent, column chromatographic[8] and in recent years high-performance liquid chromatographic (HPLC) techniques have also been applied to the separation of PLs[9]. The methods range from the analysis of PL molecular species with reversed-phase columns[10,11] to investigations that deal with the analysis of major PL classes using either gradient[12,13] or isocratic elution systems[14,15].

For example, Nissen and Kreysel[16] used a silica gel column and a polarity gradient consisting of acetonitrile and water and were able to separate C, PI, PS, PE, PC and sphingomyelin (S); however, baseline separation was not easily or reliably established. Therefore, with their method efficient collection of the PL fractions was not possible. In contrast. Hurst and Martin[17] investigated an isocratic mobile phase with acetonitrile and methanol containing 1% phosphoric acid on a silica column. This method was developed to separate PL from soya extracts where cardiolipin was not to be isolated. Using this method, cardiolipin is eluted with the solvent front. In general, the high proportion of phosphoric acid in the mobile phase causes technical problems with valve systems. Hence, the methods described to date are inappropriate for the one-step separation of PLs in complex tissues such as muscle.

In this paper we describe an HPLC method for the separation of all major PL classes from biological material and the subsequent accurate gas chromatographic (GC) determination of individual PL-bound fatty acids.

EXPERIMENTAL

Reagents, standards and apparatus

All chemicals were purchased from Merck (Darmstadt, F.R.G.) and PL standards and fatty acid methyl ester (FAME) standards from Sigma (Taufkirchen, F.R.G.). HPLC analysis of PLs was performed on a Merck–Hitachi HPLC system (655A liquid chromatograph, 655A variable-wavelength UV monitor and 655A processor), coupled with a 25 cm × 0.4 cm I.D. Si 60 (5 μm) cartridge (LiChrocard, Merck) and a fraction collector (Model 201, Gilson, Villiers-le-Bel, France). GC analysis of FAMEs was achieved using a Hewlett-Packard (Waldbronn, F.R.G.) system (5890 gas chromatograph, split injection, flame ionization detector, 7673 A autosampler, 3393A integrator), equipped with a capillary column (Durabond-Wax, 0.15 μm, 30 m × 0.25 mm I.D.; ICT, Frankfurt, F.R.G.). TLC was performed with Merck pre-coated silica gel 60 F_{254} plates (20 cm × 20 cm; 0.25 mm layer).

Tissue samples and extraction of total lipids

Total lipids were extracted from musculus longissimus dorsi (m.l.d.) near the thirteenth rib, from musculus semimembranosus (m.s.) and from the left heart ventricle (h.v.) of eighteen pigs (average mass 40 kg) using the procedures described by Folch *et al.*[18] and Hallermayer[19]. Immediately after slaughtering, samples were weighed (approximately 5 g) in a 100-ml centrifuge tube and quickly homogenized

with 35 ml of chloroform–methanol (1:1, v/v). Following centrifugation for 5 min at 4000 *g*, the supernatant was collected in a graded extraction burette. This procedure was repeated twice. To the combined supernatants 25 ml of 0.58% sodium chloride solution were added and mixed by inversion. This resulted in a clear two-phase separation with the lower chloroform phase containing the total lipids. An aliquot of the solution containing the lipids was evaporated under nitrogen at 35°C. The resulting total lipid extract was then ready for further analysis.

HPLC analysis

Total lipid extracts were diluted in chloroform–methanol (2:1, v/v) and 20 μl containing 2 mg of each sample were injected directly into the HPLC unit. Peaks were identified by comparison with the retention times of standard PLs and verification was accomplished by co-injection with standards. A solvent system was established that was based on the simultaneous use of a pH gradient and a polarity gradient. This system consisted of several steps: (1) elution with acetonitrile for 5 min; (2) elution with acetonitrile containing 0.2% phosphoric acid for 10 min; and (3) between the 15th and 25th min elution with acetonitrile containing 0.2% phosphoric acid changing continuously to methanol containing 0.2% phosphoric acid. These changes to the mobile phase were made without interruption of the flow. The flow-rate was constant at 1 ml/min. The eluted lipids were detected at 205 nm at room temperature. The PL fractions were collected automatically (electronic peak detection) and then prepared for further analysis. Elution times varied only within 3%. Complete equilibration of the system was achieved within 30 min. The purity of the individual PL fractions was checked by TLC on silica gel plates using chloroform–methanol–water (75:22:3, v/v/v) as the solvent.

Transesterification

The PL-bound FAs were converted into FAMEs according to the procedure of Shehata *et al.*[20]. The single PL fractions were evaporated from the mobile phase and 0.5 ml of a solution of 0.5 *M* methanolic sodium methylate, light petroleum and diethyl ether (1.5:6:2.5, v/v/v) was added. The solution was gently mixed and left at room temperature for 3 min. Using this procedure, transesterification of the fatty acids was determined by TLC to be nearly 100%. With S, complete esterification can only be performed by the boron trifluoride method or appropriate acidic methanolysis[21].

Gas chromatography

A 2-μl sample of each PL fraction was automatically injected into the gas chromatograph with a splitting ratio of 1:50. The temperature program used started at 100°C, was increased at 10°C/min to 240°C, and then held for 3 min at 240°C. The injection port temperature was 240°C and the detector temperature 250°C. The hydrogen flow-rate in the column was 1.8 ml/min. Peaks were identified by comparison with standards and by a common spiking procedure[22]. The amount of each fatty acid was calculated using an internal standard method; C_{17} was selected as the internal standard because in several test runs, of all PLs from the meat samples none of this fatty acid could be detected.

Phosphorimetry

Organic phosphorus was determined in the total lipid extracts by the micro method of Gentner *et al.*[23]. About 50 μg of total lipid extract were oxidized with 100 μl of concentrated sulphuric acid. Continuous heating was applied and a black colour was obtained. Next the sample was allowed to cool and 60 μl of perchloric acid (70%) were added. This mixture was then heated for 5 min, after which it was clear or slightly yellow. The sample was cooled and 2.15 ml of a mixture of 28 ml of water (doubly distilled), 5 ml of 3 *M* sulphuric acid and 10 ml of 2.5% (w/v) ammonium heptamolybdate were added. The colour reaction involved the addition of 0.25 ml of concentrated Fiske–Subbarow reagent[24] and heating at 100°C for 7 min. Finally, photometric detection was performed at 830 nm after cooling the sample to room temperature. The phosphorus concentration was determined by comparison with a calibration graph.

Calculations

Individual FAME and PL concentrations were expressed as a percentage of the total. All extractions and HPLC and GC runs were performed in duplicate and mean values were calculated. These values were used for further data processing, including analysis of variance and Student's *t*-test. The amount of each PL in each sample was calculated using the following set of equations:

(1) Determination of the total FA weight of the PL (μg):

$$x\ \mu g_{FA_1} + x\ \mu g_{FA_2} + \ldots + x\ \mu g_{FA_i} = \mu g_{\Sigma FA}$$

(2) Calculation of the relative amount of a single FA (%):

$$\frac{\mu g_{FA_1}}{\mu g_{\Sigma FA}} = \%\ FA_1; \quad \ldots \quad ; \frac{\mu g_{FA_x}}{\mu g_{\Sigma FA}} = \%\ FA_{xi}$$

(3) Calculation of average FA molecular weight (ΦMW_{FA}) of the PL:

$$\begin{array}{lll} \%\ FA_1 & \cdot\ MW_{FA_1} & = \ldots \\ \vdots & \vdots & \vdots \\ \%\ FA_i & \cdot\ MW_{FA_i} & = \ldots \\ \hline \Sigma = 100\% & & \Sigma = \Phi MW_{FA} \end{array}$$

(4) Determination of PL molecular weight (MW_{PL}):

$$n \cdot \Phi MW_{FA} + MW_{PL\,core} = MW_{PL}$$

where n = number of FA linked to the PL; ΦMW_{FA} = average FA molecular weight; $MW_{PL\,core}$ = molecular weight of a PL core which does not have FAs bonded to it.

(5) Calculation of the relative amount of FAs in the whole PL molecule (% FA_{PL});

$$\frac{n \cdot \Phi MW_{FA} \cdot 100}{MW_{PL}} = \% \, FA_{PL}$$

(6) Determination of the absolute amount of PL:

$$\frac{\mu g_{\Sigma FA}}{\% \, FA_{PL}} \cdot 100 = \text{absolute amount of PL in } \mu g/mg \text{ total extract}$$

RESULTS

A baseline separation of C, PI, PS, PE, PC, S, lyso-P-ethanolamine (LPE) and lyso-P-choline (LPC) was achieved in less than 50 min. Figs. 1 and 2 show the clear separation of these PLs. Hence, automatic fraction collection was possible, which allowed further analysis of the individual PLs by GC. Triglycerides and other non-polar lipids eluted with the solvent front and did not disturbe the PL separation. The retention times of the individual PLs were highly reproducible [coefficient of variation (C.V.) < 1%], and C.V.s of the peak areas in the linear range were between 0.8% (PC) and 3.8% (S).

The resolution of this method makes the quantitative comparison of individual PL contents possible. For example the PL composition of total lipid extracts of different tissues of pigs were found to be significantly different (Table I). PC and PE were the predominant fractions. The LPC and LPE fractions represented the plasmalogen content of the respective PIs. The lysis was assumed to be partly an effect of the low pH of the mobile phase.

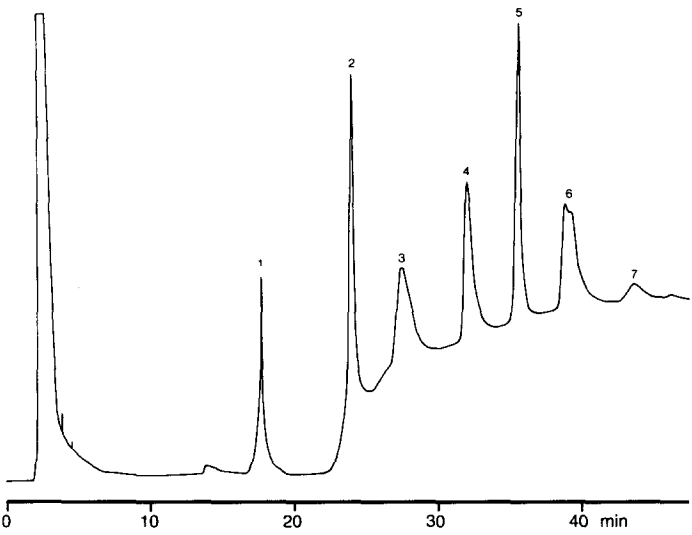

Fig. 1. HPLC of phospholipid standards: 1 = 4 μg cardiolipin; 2 = 5 μg P-inositol; 3 = 5 μg P-serine; 4 = 4 μg P-ethanolamine; 5 = 6 μg lyso-P-ethanolamine; 6 = 5 μg P-choline; 7 = 3 μg sphingomyelin.

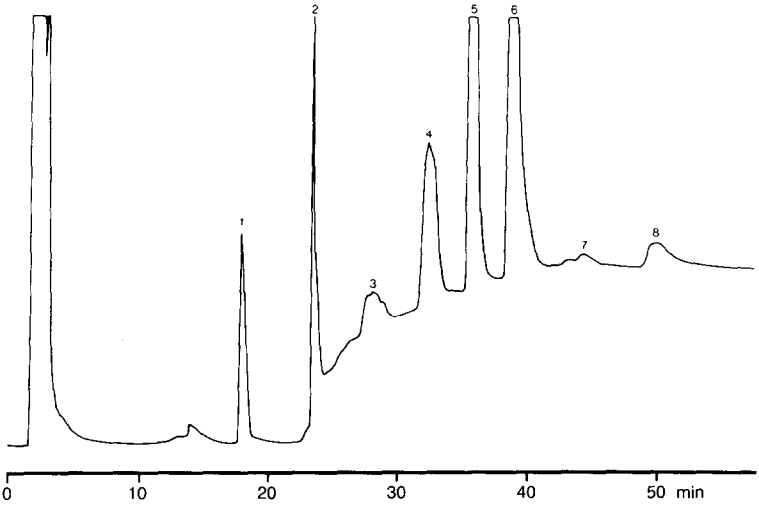

Fig. 2. HPLC of a phospholipid separation from pig muscle extract (m.l.d.); 1 = cardiolipin; 2 = P-inositol; 3 = P-serine; 4 = P-ethanolamine; 5 = lyso-P-ethanolamine; 6 = P-choline; 7 = sphingomyelin; 8 = lyso-P-choline.

The GC analysis of FAMEs was completed in 15 min. The elution of the FAMEs corresponded to their chain length and the number of double bonds within each molecule (Figs. 3 and 4). The retention times and the evaluation of the quantitative internal standard method were highly reproducible: the C.V.s of peak amounts were

TABLE I

PHOSPHOLIPID COMPOSITION (%) OF THE TOTAL LIPID EXTRACTS OF DIFFERENT TISSUES OF PIGS (n = 18)

Phospholipid	*Parameter*[a]	*m.l.d.* *(a)*	*m.s.* *(b)*	*h.v.* *(c)*	*a:b*[b]	*a:c*[b]	*b:c*[b]
Cardiolipin	LSM	16.20	12.40	13.80			
	SE	1.80	1.84	1.78			
P-Inositol	LSM	10.10	8.20	7.20	*	*	
	SE	0.60	0.60	0.58			
P-Serine	LSM	8.40	7.30	5.70		*	
	SE	0.65	0.65	0.63			
P-Ethanolamine	LSM	11.30	12.60	17.50		**	*
	SE	1.13	1.13	1.10			
Lyso-P-ethanolamine	LSM	11.00	11.10	14.70		**	**
	SE	0.90	0.90	0.90			
P-Choline	LSM	27.30	30.60	19.20		*	**
	SE	2.21	2.21	2.14			
Sphingomyelin	LSM	6.70	7.90	6.30			
	SE	0.96	0.96	0.93			
Lyso-P-choline	LSM	9.00	9.90	15.60		*	*
	SE	1.20	1.20	1.16			

[a] LSM = least-square mean; SE = standard error.
[b] * $P < 0.05$; ** $P < 0.01$.

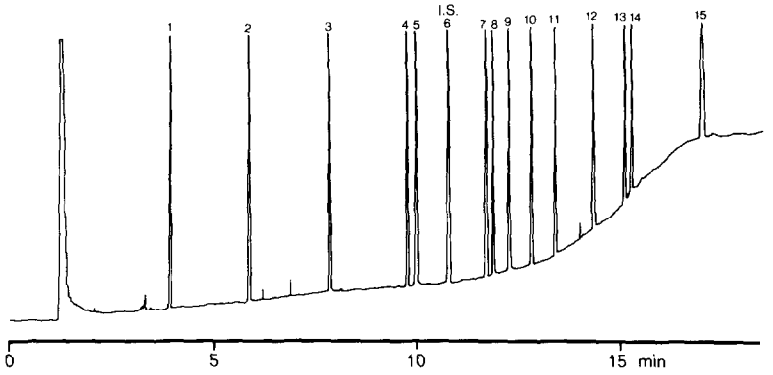

Fig. 3. Separation of FAME standards by GC: 1 = $C_{10:0}$; 2 = $C_{12:0}$; 3 = $C_{14:0}$; 4 = $C_{16:0}$; 5 = $C_{16:1}$; 6 = $C_{17:0}$ [internal standard (I.S.)]; 7 = $C_{18:0}$; 8 = $C_{18:1}$; 9 = $C_{18:2}$; 10 = $C_{18:3}$; 11 = $C_{20:0}$; 12 = $C_{20:4}$; 13 = $C_{22:0}$; 14 = $C_{22:1}$; 15 = $C_{24:0}$. Each peak represents 30 ng of substance.

between 0.2% ($C_{18:0}$) and 1.1% ($C_{14:0}$). Quantitation of the FA composition of different PLs was therefore possible. For example, the FA compositions of the same PL isolated from different tissues were significantly different (Table II). The predominant FAs of PLs isolated from the musculus longissimus dorsi and the cardiac muscle were $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$ and $C_{20:4}$.

Phosphorimetry showed a PL content in the total lipid extract of 41.4% for the musculus longissimus dorsi, 39.8% for the musculus semimembranosus and 66.7% for the cardiac muscle. In comparison, the PL contents in the total lipid extracts calculated via FAs were 36.8% for the musculus longissimus dorsi, 38.7% for the musculus semimembranosus and 71.2% for the cardiac muscle.

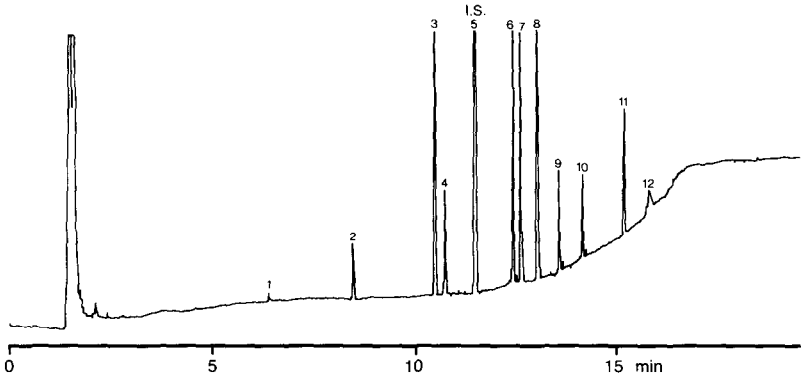

Fig. 4. GC separation of FAMEs from the P-choline fraction of pig muscle (m.l.d.) after transesterification: 1 = $C_{12:0}$; 2 = $C_{14:0}$; 3 = $C_{16:0}$; 4 = $C_{16:1}$; 5 = $C_{17:0}$ (I.S.); 6 = $C_{18:0}$; 7 = $C_{18:1}$; 8 = $C_{18:2}$; 9 = $C_{18:3}$; 10 = $C_{20:0}$; 11 = $C_{20:4}$; 12 = $C_{22:0}$.

TABLE II

FATTY ACID COMPOSITION (%) OF CARDIOLIPIN AND P-ETHANOLAMINE ISOLATED FROM MUSCULUS LONGISSIMUS DORSI (m.l.d.) AND LEFT HEART VENTRICE (h.v.) OF THE PIG

Fatty acid	*Cardiolipin*			*P-Ethanolamine*		
	m.l.d.	*h.v.*	*Diff.*[a]	*m.l.d.*	*h.v.*	*Diff.*[a]
10:0	0.2	0.5		0.1	1.0	
12:0	0.2	0.0		0.2	0.2	
14:0	2.5	2.1		1.2	0.6	
16:0	20.3	7.8	**	8.3	7.5	
16:1	3.5	4.8		1.1	0.8	
18:0	10.0	6.6		33.7	33.6	
18:1	24.4	11.5	**	14.7	7.8	**
18:2	32.3	61.1	**	20.8	15.2	**
18:3	0.9	1.7		1.0	1.1	
20:0	2.1	0.6	*	0.7	0.4	
20:4	3.2	2.6		16.6	30.7	**
22:0	0.3	0.2		0.5	0.5	
22:1	0.0	0.3		1.0	0.5	
24:0	0.0	0.1		0.1	0.2	

[a] * $P < 0.05$; ** $P < 0.01$.

DISCUSSION

The method described here for the isolation of PLs and the subsequent determination of FA contents has several major advantages. It allows one to work in a preparative manner and to collect all peaks with the necessary high purity for further quantitative analysis. The very easy preparation of the samples eliminates time-consuming pre-cleaning or pre-separation steps with the total lipid extracts. There is also no need for any derivatization procedures for increased detector sensitivity. The total PL separation can be carried out in a closed system with only one column and without further technical effort. Plasmalogen derivatives of choline and ethanolamine can be determined automatically because the corresponding lyso products are produced at low pH of the mobile phase, as proposed by Kawasaki *et al.*[25]. Therefore, no subsequent hydrolysis with concentrated hydrochloric acid[26] is necessary. In order to detect naturally occurring lyso products we used first a gradient system at pH 6.8, where only after treatment with concentrated HCl fumes were lyso products detected (Fig. 5).

The coupled analysis of PL-bound FA using GC not only provides information about the average FA pattern of the individual PLs, but also allows a precise determination of the original amount of PLs. UV sensitivity also depends on the number of double bonds in the PL-bound FA molecules, hence the calibration of the HPLC–UV detection signal is only valuable in the rare case of a uniform FA pattern. Different FA patterns of PL standards and samples certainly influence the quantitative results. Other available techniques such as micro phosphorimetric methods are very time consuming, not easy to handle and show much larger variations. However, we

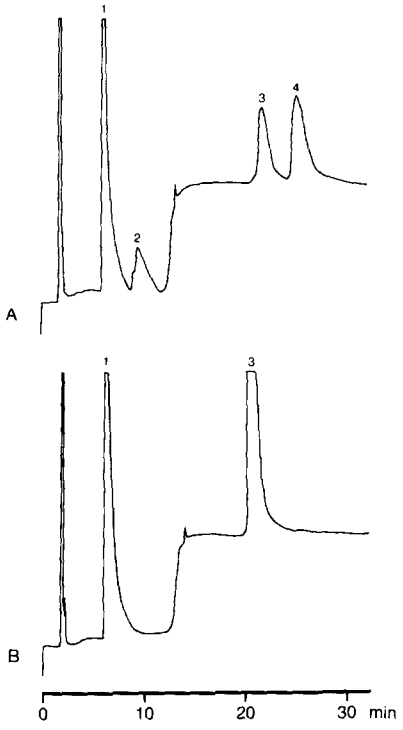

Fig. 5. HPLC of P-choline (1) and P-ethanolamine (3) and the appropriate plasmalogens lyso-P-choline (2) and lyso-P-ethanolamine (4) after treatment (A) with and (B) without concentrated HCl fumes. Mobile phase, initially 100% acetonitrile, changing between the 8th and 11th min to methanol–water (9:1). Flow-rate, constant at 1 ml/min. Lipids were detected at 205 nm at room temperature after separation on a 12.5 cm × 0.4 cm I.D. (5 μm) NH_2-cartridge (LiChrocard).

found a good correlation ($r = 0.85$) between the calculated amounts of PLs from HPLC–GC and those obtained by phosphorimetry. Nevertheless, the recalculation of the PL content from the FA pattern appears to be more precise and useful in many instances.

It was possible to work in a preparative manner and to use large amounts of samples without technical problems. The very small amount of phosphoric acid in the mobile phase did not cause valve sticking or sealing problems and in general the overall column stability was high.

A further advantage of the method described here is the possibility to easily modify the method for specific needs. For example, if the user is more interested in the PLs which elute late, such as PC and PE, there is the possibility of creating a gradient with an earlier polarity shift and/or a lower pH. If there is more interest in PLs which elute early, such as C and PI, the gradient can simply be shortened in the second half in order to save time.

Finally, the amounts and patterns of PLs and FAs from different tissues of pigs obtained by our method were comparable to the results obtained by other workers using different methods[27].

In conclusion, the procedure described is a sensitive, rapid and flexible method for the determination of PLs and FAs in mammalian muscle and can easily be adapted to other biological materials.

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