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Short communication

Method development for the separation of phospholipids by subcritical fluid chromatography

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Abstract

A packed-column subcritical fluid chromatographic separation of five phospholipids was achieved in less than 20 min by using a Luna octyl column (25 cm×4.6 mm I.D., 5 μm) employing evaporative light scattering detection. The chromatographic conditions used for the separation of (a) 1,2-dipalmitoyl-*sn*-glycero-3-phosphate (DPPA), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), (c) poly(ethylene glycolated)-1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPE–PEG), (d) 1,2-dicaproyl-*sn*-glycero-3-phosphocholine (DCPC) and (e) 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (DPPG) were: 9% ethanol–methanol (50:50, v/v), containing 0.10% (v/v) trifluoroacetic acid for 2 min, increasing by 0.5%/min to 15% in 14 min, to 40% at 14.1 min, at a column temperature of 70°C, an outlet pressure of 135 bar and a mobile phase flow-rate of 2.0 ml/min. The separation was shown to be significantly influenced by the presence of a mobile phase acidic additive (e.g. trifluoroacetic acid) and its concentration, the overall modifier ramp rate and the column outlet pressure.

Keywords: Subcritical fluid chromatography; Phospholipids

1. Introduction

Phospholipids are commonly found in plant and animal tissue and serve as structural components in membranes in addition to playing a role in enzyme activation [1]. For this reason, both their biochemical and functional activities as related to their molecular structure are explored widely. Most commonly, phospholipid mixtures are used as emulsifying additives, thus, they have found many uses in the foods, cosmetics and pharmaceutical industries [2].

Phospholipid separations are most commonly performed by thin-layer chromatography (TLC) and normal-phase high-performance liquid chromatography (HPLC). TLC is traditionally used to qualitatively separate classes of phospholipids, but the quantitative separation of individual species is a common problem [3]. Several reports have been published that describe successful separations of individual phospholipid species by HPLC [4–7]. Most separations, however, are limited by the mode of detection. For example, when UV is utilized, low wavelengths (~200 nm) must be chosen or analyte derivatization is necessary. In a few instances, evaporative light scattering detection (ELSD) has been utilized. In this regard, Olsson et al. [4]

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separated, via HPLC–ELSD, phosphatidylethanolamine, phosphatidylinositol, phosphatidic acid and phosphatidylcholine from extracted brain tissues on a Nucleosil Diol packed column with a linear mobile phase gradient consisting of a mixture of hexane–2-propanol–*n*-butanol–tetrahydrofuran–isooctane–water and a second mixture of 2-propanol–*n*-butanol–tetrahydrofuran–isooctane–water.

In 1992, Lafosse et al. [8] reported on the use of evaporative light scattering detection for the analysis of pharmaceuticals by both liquid and supercritical fluid chromatography (SFC). In this report, the first analysis of phospholipids by SFC–ELSD was described briefly. The separation of phosphatidylcholine, phosphatidic acid, phosphatidylinositol and phosphatidylethanolamine from soya lecithin was isocratically achieved in 22 min on a Zorbax silica column (25 cm×4.6 mm, d_p=5 μm) with a mobile phase consisting of carbon dioxide modified with a mixture of methanol–water–triethylamine (95:4.95:0.05, v/v/v) in a 78.4:21.6 (w/w) ratio at a column outlet pressure of 278 bar, a temperature of 45°C and a mobile phase flow-rate of 4.3 ml/min. The only SFC parameter specifically discussed in this paper was column temperature. Increased detector sensitivity and reduced analysis time were achieved by working at a low temperature, 30°C. Thus, the mobile phase was subcritical as opposed to supercritical since the critical temperature of the mixed mobile phase was above 30°C.

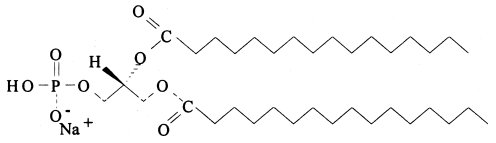
The main goal of our study was to demonstrate further the applicability of SFC to phospholipid analysis. Specifically, the objective was to develop a qualitative analytical SFC method for the separation of five phospholipids varying in polarity and ionic nature. Structures for these compounds can be found in Fig. 1. This work differs from the work described by Lafosse in that a systematic method development approach was undertaken to examine the effect of several SFC parameters on peak resolution and peak shape. The parameters investigated were: stationary phase composition, acidic modifier additive concentration, modifier ramp rate and the column outlet pressure. In contrast to the Lafosse work, the fatty acid substituents of each phospholipid were well characterized in this study. For example, phosphatidylcholine, as assayed by Lafosse, contained mostly palmitic acid (16:0) or stearic acid (18:0) in the *sn*-1 position, and unsaturated C_{18} fatty acids, such as oleic, linoleic or linolenic acid, in the *sn*-2 position [1]. In our separation, the fatty acid substituents of the choline-substituted phospholipid were known to be palmitic acid in both the *sn*-1 and *sn*-2 positions. As in the work of Lafosse, our study involves subcritical fluid chromatography, which hereafter in this manuscript will be denoted as subFC.

2. Experimental

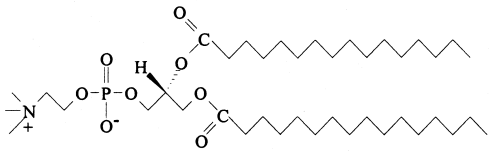
A Gilson SFC system (Middleton, WI, USA), consisting of a model 308 liquid CO_2 pump, a 306 modifier pump, a 233 XL injector, a 811 C dynamic mixer, a 831 column oven and an 821 pressure regulator, was used for all separations. An Alltech Varex Mark 11 ELSD (Deerfield, IL, USA) modified earlier for SFC was used as the detector [9]. All data were collected and analyzed using Gilson Unipoint software. All phospholipid solutions were prepared at a concentration of 1.0 mg/ml in methanol. The injection volume in all cases was 25 μl. The column effluent was split between a variable pressure controlling restrictor and the ELSD using a three-way union. The effluent was introduced to the ELSD using a silica capillary (2–3 m, 50 μm I.D.). The ELSD conditions were as follows: CO_2 gas flow-rate entering the detector, 0.3 l/min; nitrogen flow-rate, 0.7 ml/min and drift tube temperature, 70°C. The split ratio (ELSD–Variable restrictor) was 1:3. The columns investigated in this study were: (a) Valuepak Amino, 15 cm×4.6 mm, 5 μm (Keystone Scientific, Bellefonte, PA, USA), (b) Deltabond Cyano, 25 cm×4.6 mm, 5 μm (Keystone Scientific), (c) Hypersil Silica, 25 cm×4.6 mm, 5 μm (Keystone Scientific) and (d) Luna Octyl, 25 cm×4.6 mm, 5 μm (Phenomenex, Torrance, CA, USA). All chromatographic conditions can be found in the figure captions.

SFE/SFC-grade carbon dioxide without helium headspace was obtained from Air Products and Chemicals (Allentown, PA, USA). HPLC-grade methanol, ethanol and trifluoroacetic acid were purchased from EM Science (Gibbstown, NJ, USA), Aaper Alcohol (Shelbyville, KY, USA) and Sigma Aldrich (St. Louis, MO, USA), respectively. All phospholipids were donated by the DuPont Merck Pharmaceutical Company but they were originally

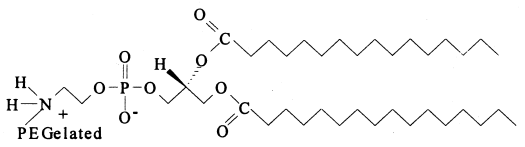
1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphate
(DPPA)



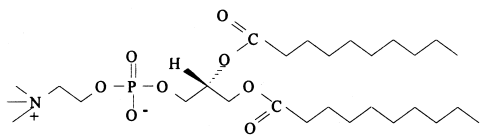
1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine
(DPPC)



1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphoethanolamine-N-[Poly(ethylene glycol)]
(DPPE-PEG)



1,2-Dicaproyl-*sn*-Glycero-3-Phosphocholine
(DCPC)



1,2-Dipalmitoyl-*sn*-Glycero-3-[Phospho-rac-(1-glycerol)]
(DPPG)

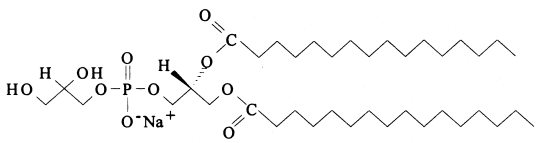


Fig. 1. Chemical structures of the phospholipids investigated.

obtained from Avanti Polar Lipids (Alabaster, AL, USA). No further purification was performed.

3. Results and discussion

Since subFC best emulates normal-phase chromatography, several normal-phase packed columns were first investigated. They were: (a) Valuepak Amino, (b) Deltabond Cyano and (c) Hypersil Silica. The subFC conditions were: 230 bar CO_2, 40°C, 2.0 ml/min liquid CO_2, 40% (v/v) methanol-modified CO_2. No peaks were observed for each singly injected phospholipid after a run time of 60 min, thus indicating that 40% methanol-modified CO_2 did not have sufficient solvent strength to elute the phospholipids from these highly active stationary phases. Earlier experiments in our laboratory had shown that

each phospholipid indeed had appreciable solubility in 40% methanol-modified CO_2.

Due to the lipophilicity of the phospholipids, the separation was next attempted on a reversed-phase column (5 μm Luna Octyl). A modifier gradient was utilized due to the varying chemical characteristics of the phospholipids investigated as well as to ensure the elution of all five analytes. The mobile phase consisted of a mixture of CO_2 and 50:50 (v/v) ethanol–methanol.

Secondary interactions between basic and acidic compounds with acidic silanols on the stationary phase surface may result in strong analyte retention and peak tailing in HPLC [10]. The addition of acidic and basic additives directly to the CO_2 modifier has been investigated in subFC in order to increase the apparent solvent strength as well as to reduce unfavorable secondary interactions [11–15]. For example, Berger et al. (cited in Ref. [5]) reported on the separation of benzene polycarboxylic acids by packed column subFC using methanol-modified CO_2, mixtures that contained a small amount of a very polar additive. Trifluoroacetic acid (TFA) was, therefore, added to the ethanol–methanol modifier (0.01%, v/v) in the hope of neutralizing the charged phosphate group on 1,2-dipalmitoyl-*sn*-glycero-3-phosphate (DPPA), as well as to reduce secondary interactions between the polar functionality on the phospholipid and any acidic silanols on the stationary phase. Only a solution of DPPA, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and poly-(ethylene glycolated)-1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPE–PEG) was injected initially. Surprisingly, only two peaks were observed in the chromatogram. The first peak was anticipated to be DPPC and DPPA, while the second peak was believed to be DPPE–PEG (Fig. 2). The peak shape of DPPC and DPPA appeared to be the same with and without the modifier additive. Later we learned upon singly injecting DPPA that the addition of trifluoroacetic acid improved the peak shape of this component. In the absence of the additive, detrimental effects on the peak shape of DPPE–PEG (i.e. decreased peak height, broadening) were, however, observed. An explanation for the additive's advantageous effect on the peak shape of DPPE–PEG may be stationary phase modification. It is well known that complete coverage of the silanol sites with the octyl groups is impossible. When introduced, the additive may preferentially absorb to these exposed silanol groups, thus reducing any interactions with the phospholipids [11].

The modifier-additive concentration was next increased from 0.01% (v/v) to 0.1% (v/v), with a modifier ramp rate of 2.0%/min, using all five components. The higher additive concentration gave

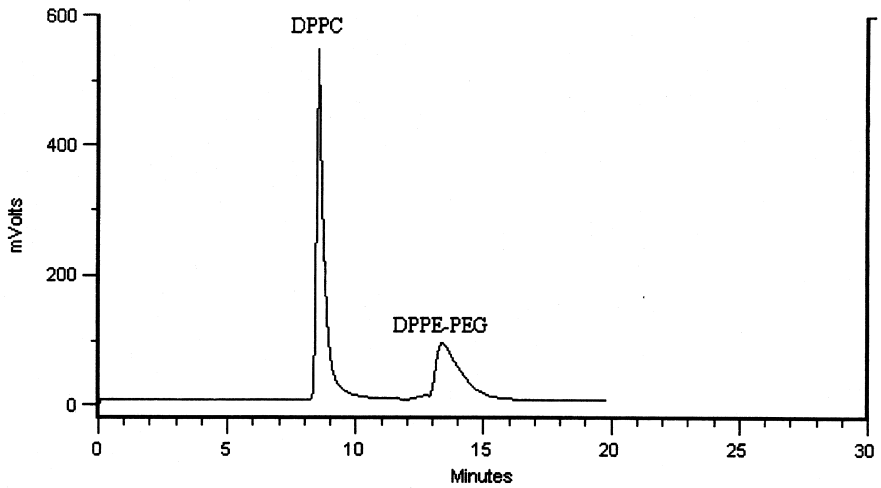


Fig. 2. Chromatogram of three phospholipids. Order of elution: DPPC (DPPA), DPPE–PEG. Mobile phase, 9% ethanol–methanol (50:50, v/v) for 2 min, ramp to 25% in 10 min (2.0%/min), 40% at 10.1 min; oven temperature, 70°C; outlet pressure, 125 bar, flow-rate, 2.0 ml/min; column, 25 cm×4.6 mm, 5 μm Luna Octyl. A 25-μg amount of each phospholipid in methanol was injected.

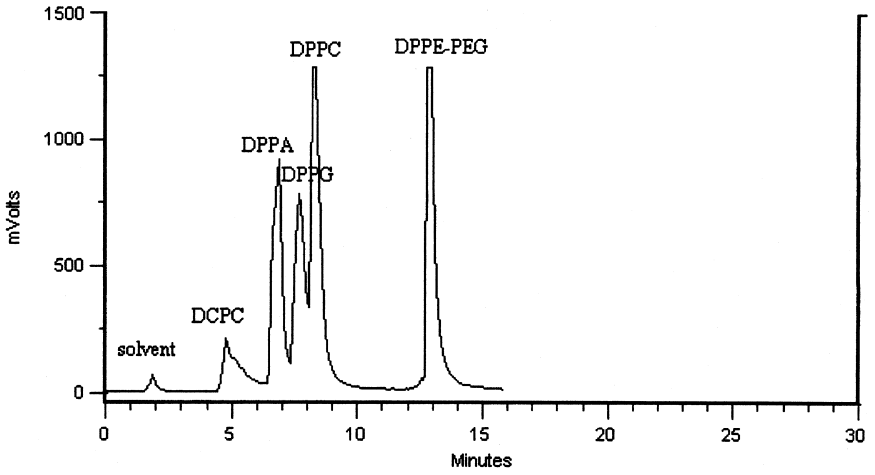


Fig. 3. Chromatogram of five phospholipids. Order of elution: DCPC, DPPA, DPPG, DPPC and DPPE–PEG. Mobile phase, 9% ethanol–methanol (50:50, v/v) containing 0.10% (v/v) trifluoroacetic acid for 2 min, ramp to 25% in 10 min (2.0%/min), 40% at 10.1 min; oven temperature, 70°C; outlet pressure, 125 bar, flow-rate, 2.0 ml/min; column, 25 cm×4.6 mm, 5 μm Luna Octyl. A 25-μg amount of each phospholipid in methanol was injected.

a definite improvement in the results (Fig. 3). Six peaks were observed in this chromatogram, including the solvent peak, 1,2-dicaproyl-*sn*-glycero-3-phosphocholine (DCPC), DPPA, 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (DPPG), DPPC and DPPE–PEG. Additionally, the peak shape/height of DPPA was further improved in the presence of a higher concentration of TFA. The selectivity of the separation was also altered since the retention of DPPG was reduced.

Similar to reversed-phase HPLC, mobile phase gradients are commonly employed in packed-column super/subcritical fluid chromatography. During the gradient, the stronger solvent, methanol, is increased in concentration at a certain rate. Although an improved separation was observed at the higher modifier-additive concentration, the resolution between DPPG and DPPC remained unsatisfactory. Simply by lowering the modifier ramp rate, baseline resolution between DPPG and DPPC was achieved. However, the peak shape of DPPA and DPPC became worse.

At this point, while the resolution between all five of the phospholipids was satisfactory, the peak shapes were less than desirable. The pressure was increased from 125 bar to 135 bar, while the modifier ramp rate was held at 0.5%/min (Fig. 4). By simply increasing the pressure by 10 bar, the peak shape and response of all of the analytes was improved without a significant loss in resolution, thereby resulting in the optimized separation of all five phospholipids. The resolution values (R_s) between the following adjacent peaks were: 1.6 (DCPC and DPPA); 1.6 (DPPA and DPPG); 1.1 (DPPG and DPPC) and 10.1 (DPPC and DPPE–PEG). Other peak parameters, including peak width at half height ($w_{1/2}$), capacity factor (k') and selectivity factor (α), for the optimized separation, can be found in Table 1. Specifically, the capacity factor (k') is a measurement that describes how long the component is retained on the stationary phase versus how long it resides in the mobile phase. Generally, k' values should be greater than one, so that there is sufficient separation from the injection solvent, and no greater than ten, where the analysis time would be too great [16]. As can be seen from the calculated capacity factors, all k' values for the phospholipid peaks ranged between one and ten. Another value that is used to evaluate the chromatographic separation is the selectivity factor (α). This value can be used to compare how close two adjacent peaks are to one another. Obviously, an α of greater than one must be achieved to ensure separation. Once again, all α values were at least 1.3. The third parameter is peak resolution (R_s). Resolution expresses how well two adjacent peaks are separated from one another.

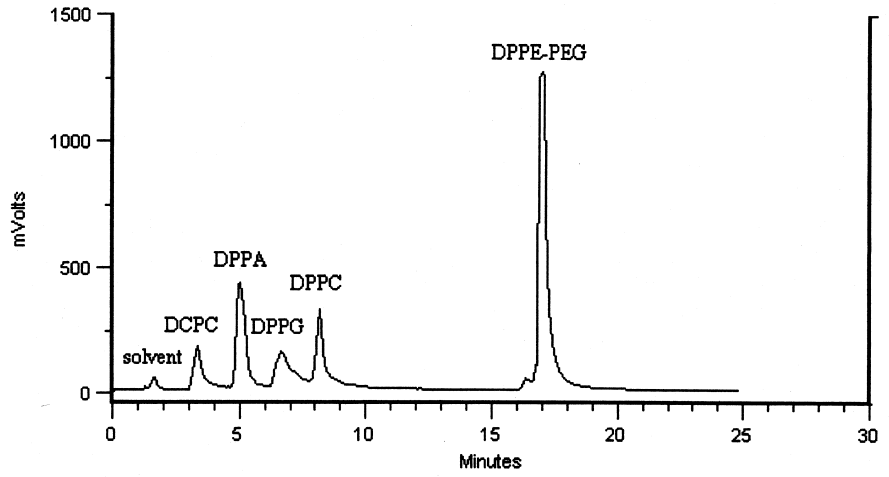


Fig. 4. Chromatogram of five phospholipids. Order of elution: DCPC, DPPA, DPPG, DPPC and DPPE–PEG. Mobile phase, 9% ethanol–methanol (50:50, v/v) containing 0.10% (v/v) trifluoroacetic acid for 2 min, ramp to 15% in 14 min (0.5%/min), 40% at 14.1 min; oven temperature, 70°C; outlet pressure, 135 bar, flow, 2.0 ml/min; column, 25 cm×4.6 mm, 5 μm Luna Octyl. A 25-μg amount of each phospholipid in methanol was injected.

Table 1
Various chromatographic peak parameters

	DCPC	DPPA	DPPG	DPPC	DPPE–PEG
Retention time (min)	3.33	5.00	6.67	8.16	16.97
T_r' (min)	1.68	3.35	5.02	6.51	15.32
$w_{1/2}$ (min)	0.33	0.40	0.62	0.29	0.32
k'	1.0	2.0	3.0	3.9	9.3
α		2.0	1.5	1.3	2.4
R_s		1.6	1.6	1.1	10.1

Mobile phase, 9% ethanol–methanol (50:50, v/v) containing 0.1% (v/v) TFA for 2 min, ramp to 15% in 14 min (0.5%/min), 40% at 14.1 min; oven temperature, 70°C; outlet pressure, 135 bar; flow-rate, 2.0 ml/min; column, 25 cm×4.6 mm, 5 μm Luna Octyl. A 25-μg amount of each phospholipid in methanol was injected.

Adequate peak separation must be ensured for quantitative purposes. Baseline separation is represented by an R_s value of 1.5, while an R_s of 1.0 indicates that the separation between the two peaks is 90% complete [16]. The separation of four of the five phospholipids resulted in baseline resolution (R_s>1.5), while the resolution between DPPG and DPPC was 1.1. Although this method was developed to serve qualitative purposes, it may prove to be a successful quantitative method due to good retention and separation in a timely manner.

Acknowledgements

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