Analysis of Phospholipids in Natural Samples by Normal Phase HPLC and Corona Charged Aerosol Detection

Marc Plante, Bruce Bailey, Ian N. Acworth
Thermo Fisher Scientific, Chelmsford, MA, USA

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Overview

Purpose: To develop HPLC methods for quantitation of phospholipids in natural products, including foods and ingredients, using an HPLC system with a charged aerosol detector.

Methods: One normal-phase HPLC method, using the Thermo Scientific™ Hypersil GOLD™ Silica column, was created for the determination of six phospholipids and was used to quantify phospholipids in foods and ingredients.

Results: Samples of foods or ingredients were analyzed for content of phospholipids using the developed method. Sensitivity for the phospholipids varied from limits of detection between 10 – 22 ng o.c.

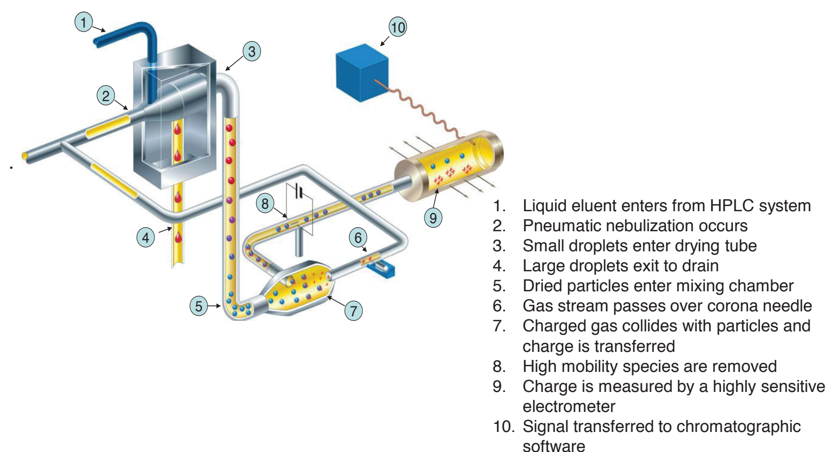
Introduction

Phospholipids are a broad class of lipids that can be divided into glycerophospholipids (GPLs) and sphingolipids. Both groups show great structural diversity. Phospholipids are amphiphilic molecules, having a hydrophilic head group, and a lipophilic fatty acid tail. Several families of GPLs exist biologically, differing in the type of polar head group present, and include: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidic acid (PA), cardiolipin, and phosphatidylinositol (PI). Each compound contains many species resulting from differences in their fatty acid composition. For example, PC may contain several different fatty ligands, which will result in mulitple peaks by reversed phase chromatography. Normal phase liquid chromatography (NP-HPLC) uses differences of polar moieties to separate analytes, and use of NP-HPLC will provide more quantitative data with less effort. Other diacyl phospholipids (e.g, dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylethanolamine (DPPE)) would exhibit similar properties. Lysophosphatides (e.g., lysophophatidylcholine (LPC)) are produced from the action of phospholipase enzymes, which removes the C2 fatty acyl side-chain.

The Thermo Scientific™ Dionex™ Corona™ Veo™ Charged Aerosol Detector, a sensitive mass-based detector, is ideally suited for the direct measurement of phospholipids, as they are non-volatile and non-chromophoric compounds. It offers excellent sensitivity (down to low nanogram amounts on column), a dynamic range of over 4 orders of magnitude, and similar inter-analyte response independent of chemical structure. This developed method is based on an original publication by Rombaut, R., et al., (J. Dairy Sci., 2005, 88, 482), that enables the direct measurement of a number of GPL and SL species, each as near-single peaks. As shown in Figure 1, the detector uses nebulization to create aerosol droplets. The mobile phase evaporates in the drying tube, leaving analyte particles, which become charged in the mixing chamber. The charge is then measured by a highly sensitive electrometer, providing reproducible, nanogram-level sensitivity. This technology has greater sensitivity, dynamic range and precision than ELSD and refractive index (RI), is gradient compatible and is simpler to operate than a mass spectrometer (MS). Compounds do not have to possess a chromophore (unlike UV detection) or be ionized (as with MS).

This sensitivity, combined with the linearity that is possible with use of the Corona Power Function, provides a unique and complete analytical solution for sensitive, reproducible, and routine analysis of non-chromophoric analytes.

FIGURE 1. Schematic and functioning of charged aerosol detection.



Methods

Liquid Chromatography

HPLC System: Thermo Scientific™ Dionex™ UltiMate™ LC System with LPG3400-SD pump (normal phase), WPS3000 RS autosampler, and TCC3000 RS column oven
HPLC Column: Thermo Scientific™ Hypersil™ Silica 3 µm, 3.0 × 150 mm
Column Temperature: 50 °C
Mobile Phase A: 0.5% diethylamine-formate, pH 3.0
Mobile Phase B: 2-Propanol
Mobile Phase C: iso-Octane
Flow Rate: 0.2–0.8 mL/min
Injection Volume: 2–5 µL
Detector: Corona Veo SD Charged Aerosol Detector
Evaporator Temperature: 50°C
Data rate: 10 Hz
Filter: 3.6 s
PowerFunction: 1.40

Flow Gradient:

Time (minutes)	Flow (mL/min)	%A	%B	%C
-4.0	0.8	1	64	35
-0.5	0.8	1	64	35
-0.2	0.2	1	64	35
0.0	0.2	1	64	35
0.1	0.8	1	64	35
2.0	0.8	4	61	35
7.0	0.8	10	60	30
13	0.8	10	60	30
14	0.8	1	64	35

Standard and Sample Preparations

Standards were dissolved in methanol / chloroform (1:1), at a concentration of approximately 2.00 mg/mL, including the sodium salt for the PI and PS analytes. Standards were diluted sequentially by half to provide a calibration range of 78 – 10,000 ng o.c. of each analyte.

Egg Yolk Sample:

In a 2 mL glass vial, 90.3 mg yolk was placed. To this, 150 uL of methanol / chloroform (1:1) was added. The mixture was vortex-mixed. To precipitate proteins, 500 µL of acetonitrile was added and the mixture was vortex-mixed. Another 1,350 µL of methanol / chloroform (1:1) was added, vortex-mixed, and allowed to settle. The supernatant was centrifuged at 10,000 g for 3 minutes, and the supernatant was analyzed directly.

Lecithin:

To an HPLC vial, 1.1 mg of Lecithin (Laboratory Grade, Fisher Scientific) was added. The sample was dissolved in 1100 µL of methanol / chloroform (1:1) and centrifuge-filtered through a 0.2 micron filter, 10,000 g for 3 minutes Supernatant was analyzed directly.

Krill Oil:

To an HPLC vial, 2.50 mg of krill oil was added. To this, 1000 µL of methanol/chloroform (1:1) was added. The solution was vortex-mixed and analyzed directly.

Data Analysis

All HPLC chromatograms were obtained and compiled using Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System software, 6.8 SR 13.

Results

Calibrations

Five phospholipids were used to calibrate the method for sample analysis: PE, PI, PS, PC, and LPC. A chromatogram of the standard solution at approximately 5000 ng o.c. is shown in Figure 2. Each analyte solution was injected threefold, plotted, and fit to a linear regression, as shown in Figure 3. As can be seen from the plot, all plots were linear, and four of the five analytes had nearly the same response factor, which is a typical characteristic of charged aerosol detectors with non-volatile analytes. Other phospholipids, PA, PG, and SPH were not included in these analyses. PA and PG eluted at the retention time of 4.8 minutes, or the small peak seen just after PE, in Figure 2.

The correlation coefficients, R^2, for all analytes was greater than 0.995 across the entire dynamic range, from 78 to over 10,000 ng. o.c. Along with these values, the sensitivity results, based on signal to noise calculations for LOD and LOQ, are provided in Table 1.

Figure 2. HPLC-CAD chromatogram overlays of phospholipid standards at 5000 ng o.c. in triplicate.

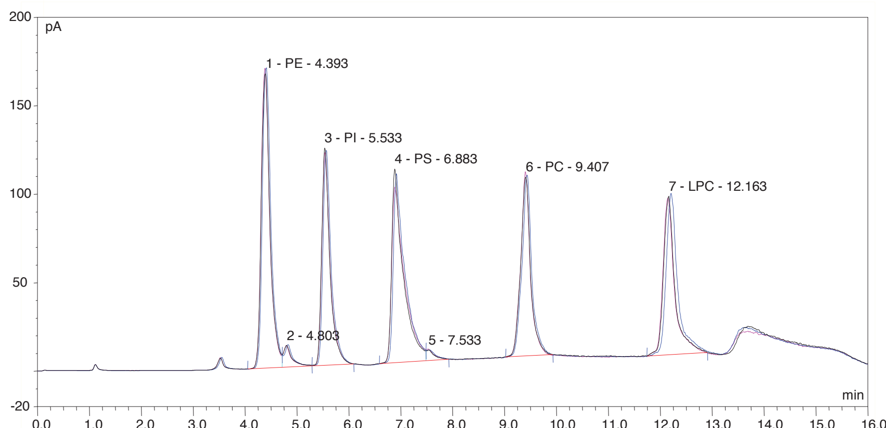


Figure 3. Linear regression calibrations curves for PE, PI, PS, PC, and LPC, from 78–10,000 ng o.c., each in triplicate.

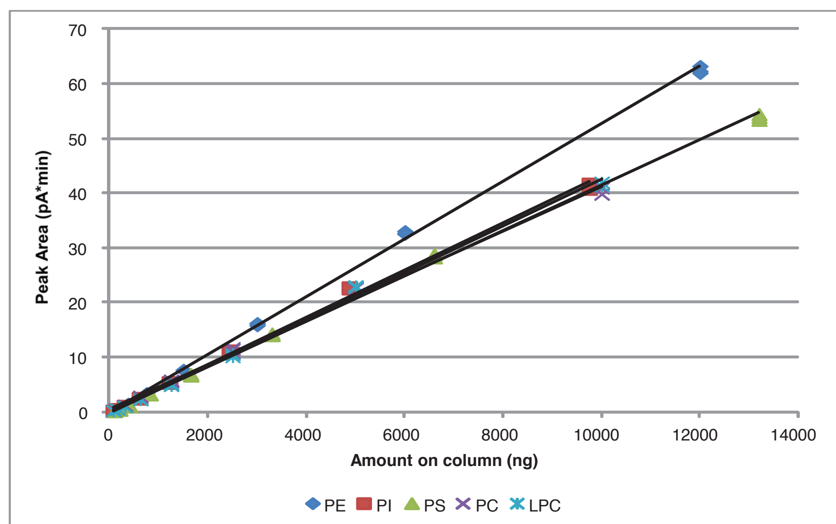


Table 1. Calibration and Sensitivity for PE, PI, PS, PC, and LPC for 78 – 10,000 ng o.c.

Phospholipid	R^2	LOD (ng o.c.)*	LOQ (ng o.c.)**
PE	0.9991	14	42
PI	0.9973	14	42
PS	0.9988	22	67
PC	0.9955	10	31
LPC	0.9974	14	41

* LOD calculated at signal to noise ratio of 3.3, at lowest on column amount for each analyte.
** LOQ calculated at signal to noise ratio of 10, at lowest on column amount for each analyte.

Sample Analysis—Egg Yolk

The sample of egg yolk was analyzed directly, as prepared, with a 2 µL injection volume. The resulting chromatogram was clean of interfering peaks, as shown in Figure 4. The compositional results[1] match literature values (obtained by extraction and ^{31}P-NMR) very well, as provided in Table 2. The actual amount of phospholipids differed from a quantitative study,[2] possibly due to differences between eggs and / or the extraction efficiency of the method used to clean the sample.

Figure 4. HPLC-CAD chromatogram showing phospholipids found in egg yolk.

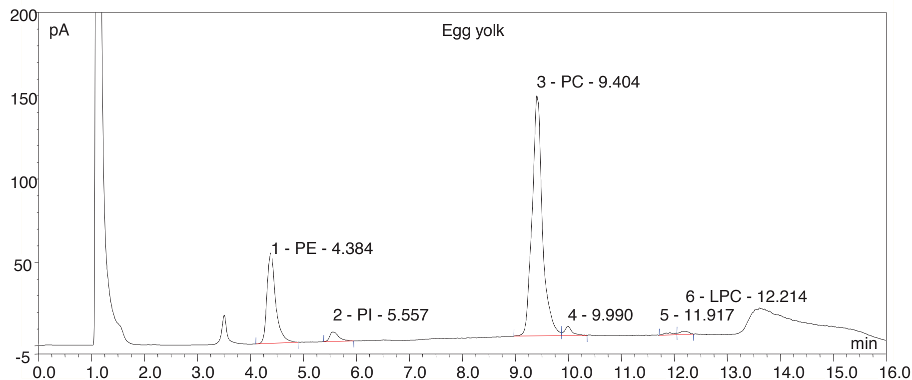


Table 2. Results of egg yolk analysis compared to literature values, including total, relative composition of total phospholipids (PL).

Phospholipid	Literature (PL-%)[1]	Found (PL-%)
PE	19.86	18.9
PI	1.73	2.22
PS	0.0	0.0
PC	75.40	75.6
LPC	0.04	0.92
Total[2]	**97.03 mg/g**	**86.6 mg/g**

Sample Analysis—Lecithin (Laboratory Grade)

The lecithin sample was analyzed directly, with chromatogram shown in Figure 5, finding PE, possibly PA (peak at 4.59 minutes), PI, PS, PC. The LPC peak retention time was obscured by matrix peak. Composition and total phospholipids results are provided in Table 3.

Figure 5. HPLC-CAD chromatogram showing phospholipids found in lecithin.

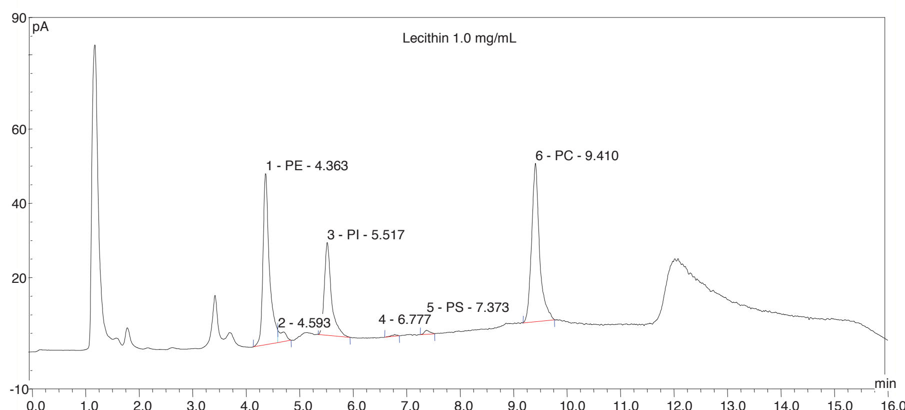


Table 3. Results of laboratory-grade lecithin analysis, including total phospholipids and mass-% of each phospholipid relative to amount of lecithin.

Phospholipid	Found (mass-%)
PE	25.2
PI	17.0
PS	1.2
PC	33.6
Total	**792 mg/g**

Sample Analysis—Krill Oil

The krill oil sample was analyzed directly, with chromatogram shown in Figure 6, finding the major phospholipids present, mainly PC, PE, and LPC and the minor PI and PS phospholipids. Relative phospholipid composition and total phospholipids results are provided in Table 4. The values obtained matched the AOCS method results within 15%.

Figure 6. HPLC-CAD chromatogram showing phospholipids found in krill oil.

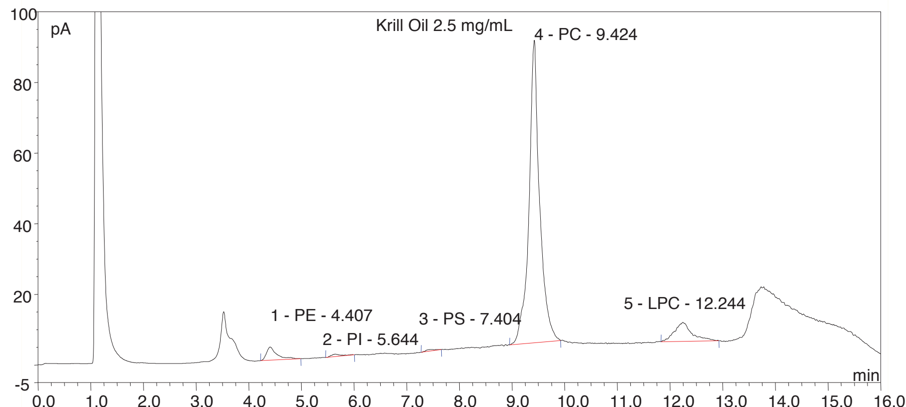


Table 4. Results of krill oil analysis compared to literature values, including total and relative composition of total phospholipids.

Phospholipid	AOCS (PL-%)*	Found (PL-%)
PE	4.6	3.5
PI	--	0.14
PS	--	1.05
PC	81.2	86.7
LPC	10.6	8.64
Total	**43.0 mg / g**	**44.1 mg/g**

***** AOCS Official Method Ja 7c-07[3]

Conclusions

A method for the determination of phospholipids in various samples was developed using normal phase HPLC and the Corona Veo Charged Aerosol Detector. Sensitivities for the phospholipids were < 25 ng o.c. LOD.

Linear calibration curves were found for all five phospholipid analytes, with correlation coefficients > 0.995 over four orders of magnitude.

Phospholipids compositional results matched orthogonal results very well, and quantity amounts were similar, whereby differences were more likely due to sample preparation rather than chromatographic method conditions.

References

1. El Bagir, N.M.; et al. Lipid Composition of Egg Yolk and Serum in Laying Hens Fed Diets Containing Black Cumin (Nigella sativa*). International Journal of Poultry Science* **2006**, *5(6)*, 574–578.

2. Walker, L.A.; Wang, T.; Xin, H.; Dokle, D. Supplementation of Laying-Hen Feed with Palm Tocos and Algae Astaxanthin for Egg Yolk Nutrient Enrichment. *J. Agric.*

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France +33 1 60 92 48 00
Germany +49 6103 408 1014
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