An Isocratic HPLC Method for the Simultaneous Determination of Cholesterol, Cardiolipin, and DOPC in Lyophilized Lipids and Liposomal Formulations*

Ninus Simonzadeh
Cobrek Pharmaceuticals Inc., 2201 W. Campbell Park Dr., Chicago, IL 60612

Abstract

Phospholipids, such as 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), and 1,1',2,2'-tetramyristoyl cardiolipin, along with cholesterol, form liposomes in aqueous media and have been investigated at NeoPharm (Lake Bluff, IL) as drug-delivery systems. To accurately assess the effectiveness of various formulations involving the use of aforementioned phospholipids and cholesterol, their quantitative determination is essential. An isocratic high-performance liquid chromatographic method for the simultaneous determination of cholesterol, cardiolipin, and DOPC in various pharmaceutical formulations containing the active drug substance has consequently been developed and is presented here. The current method utilizes an ASTEC-diol analytical column and is shown to be stability-indicating and free from interference from any of the formulation excipients, such as sucrose, sodium chloride, and sodium lactate. The analytes are detected using an evaporative light scattering detector (Alltech or Polymer Laboratories). The quantitation of each lipid component is performed using non-linear regression analysis. The retention characteristics of the analytes are examined as a function of eluent composition (e.g., pH, salt content, organic to aqueous phase ratio) and column temperature. The method was validated and was found to be sensitive, specific, rugged, and cost-effective. The current method provides enhanced chromatographic separation for lipid components as well as degradation products as compared to similar methods reported in the literature. It is also inherently simpler than other similar methods reported in the literature that typically use complex gradient elution.

Introduction

1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) and acidic phospholipids such as cardiolipin (Figure 1) form liposomes in aqueous media and are being evaluated at NeoPharm as drug-delivery systems. Cholesterol is usually included in the formulations to stabilize the liposomal membrane and to minimize leaching of the encapsulated water-soluble drugs. Among the advantages in using liposomes in pharmaceutical formulations is a lowered inherent toxicity and improved pharmacokinetic profile associated with various anti-cancer drugs.

Advantages of evaporative light scattering detector (ELSD) for the detection of phospholipids and various non-chromophoric compounds include the fact that derivatization is not required and that sample preparation and analysis time is minimized in

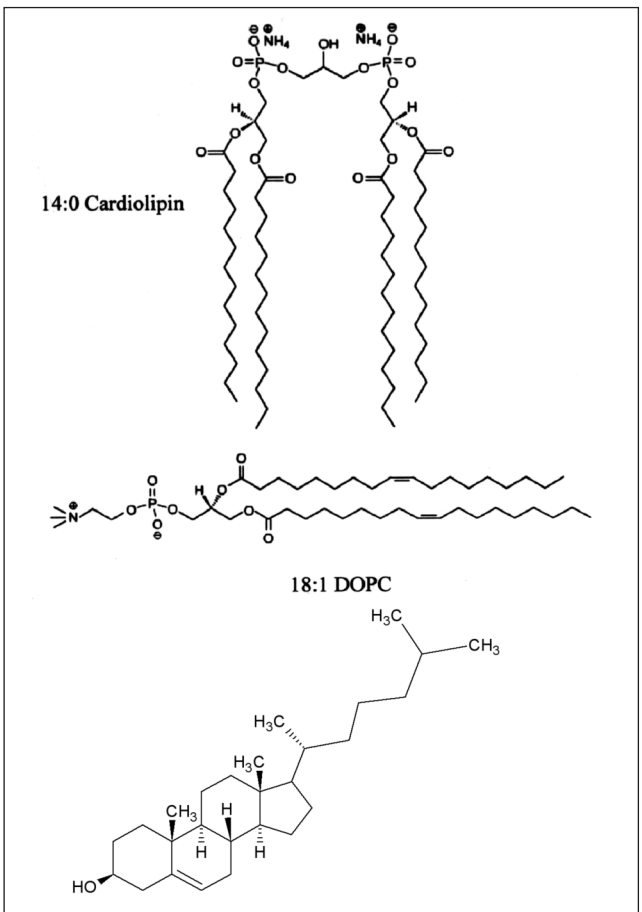

Figure 1. Structures of 14:0 cardiolipin, 18:1 DOPC, and cholesterol.

* The work described in this manuscript was performed at NeoPharm, 101 Waukegan Rd., Suite 970 Lake Bluff, IL 60044.

comparison to fluorescence detection. In comparison to UV–vis detection, solvent is not selected for spectral properties; detection is independent of absorbance characteristics, and derivatization is not required for non-chromophoric compounds. In comparison to refractive index detection, ELSD provides better sensitivity and is gradient compatible with stable baselines and no solvent front peaks.

Among the various methods that have been used to separate and quantify phospholipids are ion-exchange methods with suppressed-conductivity detection (l), high-performance liquid chromatography (HPLC) methods with UV detection (2,3), HPLC methods with light-scattering detection (4–7,8,9,10,11), HPLC methods with light-scattering and mass spectrometric (MS) detection (10), HPLC methods using a charged aerosol detector based on charging the aerosol particles and measuring the current from the charged aerosol flux in the nebulized effluent (12), gas chromatographic (GC) methods involving derivatization (13–17), and capillary electrophoretic (CE) methods (18,19). Typically, HPLC methods using conductivity and light-scattering detection have shown enhanced sensitivity in comparison with those using UV detection and are also relatively simpler than GC methods, which almost invariably include a derivatization step.

The work described here represents an alternate way to separate and quantify these lipids in various formulations using simple isocratic elution coupled with light-scattering detection. The method has been successfully applied to the analysis of lyophilized lipids and aqueous liposomal formulations containing cholesterol and phospholipids in media such as normal saline and acidic lactate buffer.

Experimental

Reagents

All reagents were of analytical grade. DOPC, cardiolipin and lysoPC were purchased from Avanti Polar Lipids (Alabaster, AL). Tocopherol succinate and oleic acid were obtained from Sigma (St. Louis, MO). Cholesterol, Standard Reference Material was purchased from National Institute of Standards and Technology (NIST, Gaithersburg, MD). Ammonium acetate and sodium lactate were obtained from Fisher Scientific (Fair Lawn, NJ). HPLC-grade chloroform (ethanol-preserved) was purchased from EM Science (OmniSolv) (Gibbstown, NJ) or from Fisher Scientific. [Note: Chloroform from EM Science (OmniPur) or from Fisher Scientific (Optima preserved with amylene) should not be used as either a mobile phase component or as solvent for sample or standard preparation.]

HPLC-grade methanol was obtained from Fisher Scientific or EM Science. Glacial acetic acid was obtained from EM Science. Myristic acid was purchased from Nu-Check Prep (Elysian, MN). Hydrochloric acid (l N) was obtained from J.T. Baker (Phillipsburg, NJ). Sucrose was purchased from Mallinckrodt (Paris, Kentucky). The various formulations analyzed in this work containing the active drug substance at 1–2 mg/mL concentration, and cardiolipin, DOPC, and cholesterol each present in the approximate concentration range of ~ 2–36 mg/mL with sucrose at 10–20% w/v, were prepared in-house and in media such as normal saline or 10mM lactate buffer (pH 1.5–2.0). Milli-Q water (18 MΩ.cm) was used to prepare all aqueous reagent solutions. The diluent used for the preparation of working standards and samples is a mixture of chloroform–methanol–water (71:26:3, v/v). Mobile phase is a premixed mixture of chloroform–methanol–aqueous ammonium acetate (71:26:3, v/v) [ammonium acetate is a 10mM solution with an optimal pH range of 9.0–9.9 adjusted with dilute (1:1, v/v) aqueous ammonium hydroxide.]

Preparation of samples and standards

Inasmuch as the regression plot for each analyte could usually be best described by a 2nd order polynomial, to enhance the accuracy of results, a minimum of ten ternary working standards were prepared and chromatographed for each analysis. Each working standard consisted of cholesterol, cardiolipin, and DOPC in the approximate concentration range of 5–110 µg/mL. The formulations examined consisted of samples containing the active drug substance in the 1–2 mg/mL concentration range, or placebo formulations with no drug present, as well as DOPC, cardiolipin, and cholesterol, each present in varying concentrations ranging from ~2–36 mg/mL. The working sample solutions were prepared by diluting each formulation (typically 400×) with diluent.

Instrumentation

The HPLC system used was an Agilent 1100 (Agilent Technologies, Inc., Santa Clara, CA) unit consisting of a binary pump or quaternary pump, vacuum degasser, column oven, thermostated auto-sampler, Agilent software (Chemstation), an HP Vectra computer and an evaporative light scattering detector [model PL-ELS1000, Polymer Laboratories (Amherst, MA), or an Alltech ELSD (Nicholasville, KY) model ELSD 2000]. The analytical column used was a 250 × 4.6 mm, 5-µm diol, catalog no. 51080 purchased from Advanced Separation Technologies, Inc. (ASTEC) (Whippany, NJ).

Nominal chromatographic conditions

The nominal chromatographic conditions were as follows: column, ASTEC diol, 250 mm × 4.6 mm, 5 µm particles, catalog

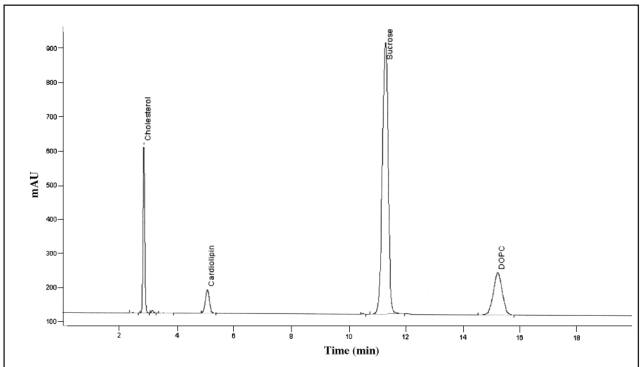

Figure 2. Chromatogram of a diluted liposomal formulation containing ~ 240 µg/mL in total lipids and 40 µg/mL in sucrose using the nominal eluent composition: chloroform–methanol–10mM ammonium acetate (pH 9.9) (71:26:3 v/v).

no. 51080; mobile phase, chloroform–methanol–aqueous ammonium acetate (10mM, pH 9.0–9.9) (71:26:3 v/v); flow rate, 1.0 mL/min; column temp., 40°C; auto-sampler temp., 5°C; injection volume, 50 µL.

The detector settings were as follows: Polymer Labs ELSD (model PL-ELS1000) Alltech ELSD (model ELSD 2000); evaporation temp., 110°C, Impactor off mode; nebulazation temp., 80°C; tube temp., 80°C; nitrogen gas flow rate, 1 L/min; nitrogen gas flow rate, 0.8 L/min; transfer line temp, 30°C.

Results and Discussion

Optimization of elution conditions

The effect of mobile phase composition on the retention time or the elution order of sample components was examined. Figure 2 shows a typical chromatogram of a working sample solution obtained under the nominal HPLC conditions described in the "Nominal chromatographic conditions" section. The variations in mobile phase composition included changes in the organic to aqueous phase ratio, changes in pH and in the salt content, and changes in the chloroform-to-methanol volume ratio. The effect of other variables such as column temperature on retention time was also studied. The variables that were observed to have a pronounced effect on the retention of the lipids were the organic-to-aqueous phase volume ratio and the chloroform-to-methanol volume ratio. For example, changing the chloroform-to-methanol volume ratio of 71:26 (v/v) to 73:24 (v/v) caused a reversal in the elution order of DOPC and sucrose (a formulation excipient, Figure 3). This is due to the difference in the relative solubility of DOPC and sucrose in chloroform and methanol and the corresponding affinity for the polar diol phase. Increasing the pH of the aqueous portion of the mobile phase from 4 to ~ 10, while maintaining a constant acetate concentration of 10mM appeared to cause a modest increase in the retention time of DOPC and that of cardiolipin starting at ~ pH 8 (Figure 4). This may be due to the deprotonation of the phosphate group in DOPC and cardiolipin coupled with possible ionization of the 2-hydroxy group of the central glycerol backbone in cardiolipin with increasing pH and the corresponding increase in the hydrophilicity of the two compounds. Increasing the acetate ion concentration from 10mM to 50mM (i.e., 0.30 to 1.5mM in the bulk eluent) did not significantly affect the retention characteristics of any of the analytes. Interestingly, however, increasing the column temperature had the effect of a modest increase in the retention of DOPC, while not significantly affecting the retention time of the other lipids (Figure 5). This is postulated to be the result of a decrease in pKa of the hydroxyl groups of the diol phase with temperature (20), and the corresponding increase in their interaction with the quaternary ammonium group of the DOPC molecule.

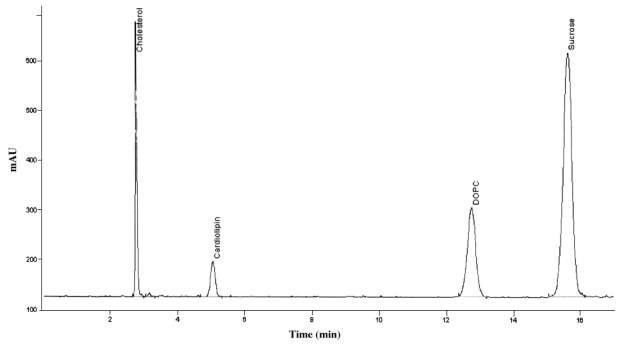

Figure 3. Chromatogram of a diluted liposomal formulation containing ~ 240 µg/mL in total lipids and 40 µg/mL in sucrose using chloroform–methanol–10mM ammonium acetate (pH 9.9) (73:24:3, v/v) as eluent.

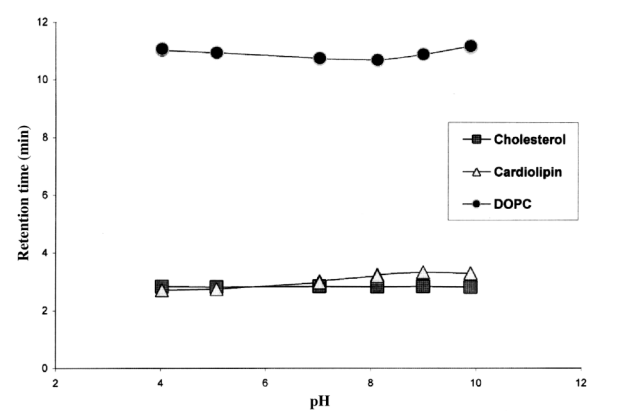

Figure 4. Effect of pH of the aqueous portion of the mobile phase on retention times of DOPC, cholesterol, and cardiolipin.

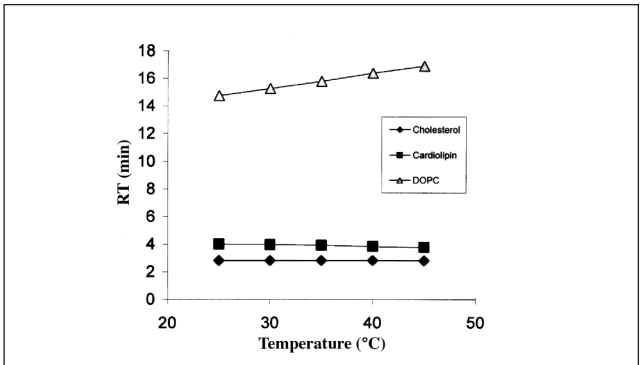

Figure 5. Effect of column temerature on retention times of DOPC, cholesterol, and cadiolipin.

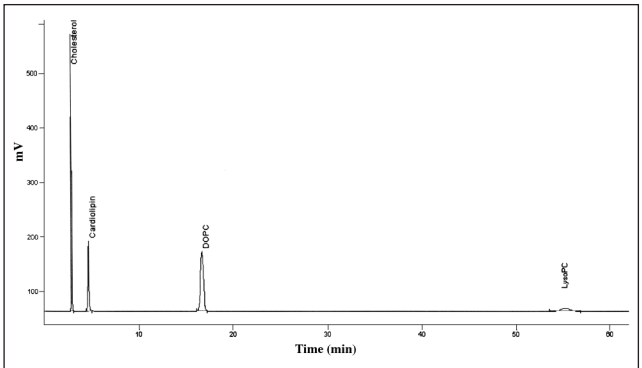

Figure 6. Chromatogram of a solution containing cholesterol, cardiolipin, DOPC, and lysoPC, each at ~ 50 µg/mL using the nominal HPLC conditions.

The diol stationary phase has been used for the separation of lipids (21–22), and when it was used in this work, provided the required separation and is known for greater stability and more rapid re-equilibration times as oppose to the non-bonded silica column (11). In normal phase mode, the diol phase is known to provide an alternative selectivity to silica, often with increased retention, which helps to improve separation power (23).

Elution of potential degradation products and excipients

Using the nominal mobile phase composition (see "Nominal chromatographic conditions" section), potential degradation products such as 18:1 lysoPC (1-oleoyl-2-hydroxy-sn-glycero-3-phosphocholine) with a relative retention time (RRT) of 3.31 vs. DOPC (Figure 6), and myristic acid or oleic acid, RRT of 1.23 and 1.16 (vs. cholesterol), respectively, do not interfere with the elution of any of the analytes. Also, none of the degradation products generated by thermal stressing or by treatment of the formulation samples with acid (TFA) or base (ammonium hydroxide) interfered with the elution or quantification of the analytes. Similarly, formulation excipients such as sucrose or vitamin E are also observed to be baseline-resolved from the analytes (Figures 1 and 7).

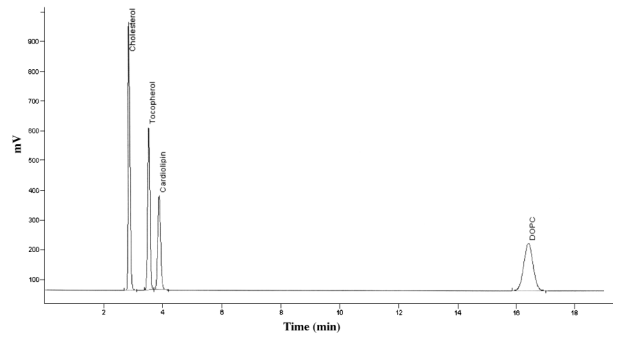

Figure 7. Chromatogram of a solution containing cholesterol, cardiolipin, DOPC, and tocopherol succinate, each at ~ 50 µg/mL.

Table I. Accuracy (Standard Addition-Recovery) Results for Cholesterol, DOPC, and Cardiolipin

Level (% of target)	Sample	% Recovery for cholesterol	% Recovery for cardiolipin	% Recovery for DOPC
80%	Prep 1	99.4	101.6	100.0
	Prep 2	99.3	103.8	100.1
	Prep 3	99.4	101.5	99.5
	Average	99.4	102.3	99.9
	SD*	0.1	1.3	0.3
	%RSD†	0.1	1.3	0.3
100%	Prep 1	99.8	101.5	100.1
	Prep 2	97.7	101.1	99.6
	Prep 3	98.5	100.4	99.0
	Average	98.7	101.0	99.6
	SD	1.1	0.6	0.6
	%RSD	1.1	0.6	0.6
120%	Prep 1	95.2	99.4	100.4
	Prep 2	99.3	102.0	98.7
	Prep 3	100.7	99.2	99.6
	Average	98.4	100.2	99.6
	SD	2.9	1.6	0.9
	%RSD	2.9	1.6	0.9

* SD = standard deviation.
† %RSD = % relative standard deviation.

Method validation

Table I lists the accuracy (standard addition-recovery) data for placebo samples spiked with known amounts of each lipid component. For example, when determining the recovery for cardiolipin, known amounts of cardiolpin were spiked into a placebo sample containing target amounts of cholesterol and DOPC as well as the excipients, such as sucrose, sodium lactate, and vitamin E succinate. Working sample solutions were prepared from the spiked samples and subsequently assayed as per conditions listed in the "Nominal chromatographic conditions" section.

The evaluation of method precision consisted of the analysis of six independent preparations of a pharmaceutical formulation containing DOPC, cardiolipin, and cholesterol in a 10mM lactate buffer (pH 2) medium. The six working sample preparations were analyzed on two consecutive days with an overall %RSD of ± 1.3, ± 1.0, and ± 1.5 for cholesterol, cardiolipin, and DOPC, respectively.

Included in the method validation experiments were those used to determine the limit of quantitation (LOQ) for the three analytes. The LOQs were determined to be 0.3, 0.6, and 0.6 µg/mL for cholesterol, DOPC, and cardiolipin, respectively.

Conclusion

An HPLC method using light scattering detection for the simultaneous determination of cholesterol, DOPC, and cardiolipin has been developed and is presented here. The method uses simple isocratic elution and is shown to be selective, providing good precision, accuracy, and sensitivity. It has been applied to the analysis of lyophilized lipids and aqueous liposomal formulations in media such as normal saline and acidic lactate buffer.

Acknowledgment

The author gratefully acknowledges Dr. Sydney Ugwu, Dr. Allen Zhang of NeoPharm for their review of this manuscript and Mr. Tommaso Sardone for his analytical support. The author also gratefully acknowledges NeoPharm for the use of the facilities, equipment and all reagents and solvents used to carry out the experiments necessary for the completion of this work.

This work was also presented at the 2002 ACS 17th Rocky Mountain Regional Meeting.

References

1. C. Nasuhoglu, S.Y. Feng, J.P. Mao, M. Yamamoto, H.L. Yin, B. Earnest, A. Barylko, J.P. Albanesi, and D.B. Hilgemann. Nonradioactive analysis of phosphatidylinositides and other anionic phospholipids by anion-exchange high-performance liquid chromatography with suppressed conductivity detection. *Anal. Biochem.* **301:** 243–54 (2002).
2. C.A. Demopoulos, T. Nomikos, H.C. Karantonis, and E. Fragopoulou. One-step separation system for the main phospholipids, glycolipids, and phenolics by normal phase HPLC. Application to polar lipid extracts from olive and sunflower. *J. Liq. Chromatogr. Rel. Tech.* **25(1):** 137–49 (2002)
3. J.H. Oak, K. Nakagawa, and T. Miyazawa. UV analysis of amadori-glycated phosphatidylethanolamine in foods and biological samples. *J. Lipid Res.* **43:** 523–29 (2002).
4. B.S. Lutzke and J.M. Braughler. An improved method for the identification and quantitation of biological lipids by HPLC using laser light-scattering detection. *J. Lipid Res.* **31:** 2127–30 (1990).
5. J. Becart, C. Chevalier, and J.P. Biesse. Quantitative analysis of phospholipids by HPLC with a light scattering detector: Application to raw materials for cosmetic use. *J. High Resolut. Chromatogr.* **13:** 126–29 (1990).
6. N.U. Olsson, A.J. Harding, C. Harper, and N. Salem. High-performance liquid chromatography method with light scattering detection for measurements of lipid class composition: Analysis of brains from alcoholics. *J. Chromatogr.* **681:** 213–18 (1996).
7. K.C. Arnoldsson and N.U. Olsson. *Analysis of Polar Lipids with HPLC and Light-Scattering Detection in Phospholipids: Characterization, Metabolism, and Novel Biological Applications.* G. Cevc and F. Paltauf, Eds., AOCS Press, Champaign, IL, 1995, 44–55.
8. R.A. Moreau. "The Evaporative Light-Scattering Detector as a Tool for the Analysis of Lipids by HPLC". J.-T. Lin & T.A. McKeon, Eds. In *HPLC of Acyl Lipids*. H.N.B. Publishing, New York, 2005, pp. 93–116.
9 A. Avalli and G. Contarini. Determination of phospholipids in dairy products by SPE/HPLC/ELSD. *J. Chromatogr. A* **1071(1-2):** 185–90 (2005).
10. V. Ioffe, T. Kalendarev, I. Rubinstein, and G. Zupkovitz. Reverse phase HPLC for polar lipids. Simple and selective HPLC procedures for analysis of phospholipid-based derivatives of valproic acid and various non-steroidal anti-inflammatory drugs. *J. Pharm. Biomed. Anal.* **30:(3)** 391–403 (2002).
11. B.J. Stith, J. Hall, P. Ayres, L. Waggoner, J.D. Moore, and W.A. Shaw. Quantification of major classes of Xenopus phospholipids by high performance liquid chromatography with evaporative light scattering detection. *J. Lipid Res.* **41:** 1448–54 (2000).
12. R.A. Moreau. The quantitative analysis of lipids via HPLC with a charged aerosol detector. *Lipids J.* **41(7):** 727–34 (2006).
13. J. Coene, E. Van den Eeckhout, and P. Herdewijn. GC determination of alkyl lysophospholipids after solid-phase extraction from culture media. *J. Chromatogr.* **612:** 21–26 (1993).
14. D.V. Lynch and G.A. Thompson. Analysis of phospholipid molecular species by gas chromatography and coupled gas chromatography–mass spectrometry. In *Modern Methods of Plant Analysis. 3. Gas Chromatography/Mass Spectrometry*. H.S. Liskens and J.F. Jackson, Eds, Springer-Verlag, Berlin, 1986 pp. 100–120.
15. P.D. Nichols, P.M. Shaw, C.A. Mancuso, and P.D. Franzmann. Analysis of archaeal phospholipid-derived di- and tetraether lipids by high temperature capillary GC. *J. Microbial Meth.* **18:** 1–9 (1993).
16. N.U. Olsson and P. Kaufmann. Optimized method for the determination of 1,2-diacyl-sn-glycero-3- phosphocholine and 1,2-diacyl-sn-glycero-3-phosphoethanolamine molecular species by enzymatic hydrolysis and GC. *J. Chromatogr.* **600:** 257–66 (1992).
17. C. Yon and J.S. Han. Analysis of trimethylsilyl derivatization products of phosphatidylethanol by gas chromatography–mass spectrometry. *Exp. Mol. Med.* **32:** 243–245 (2000).
18. L. Zhang, S. Krylov, S. Hu, and N.J. Dovichi. Methyl-β-cyclodextrin modified micellar electrokinetic capillary chromatography with laser-induced fluorescence for separation and detection of phospholipids. *J. Chromatogr.* **895:** 129–134 (2000).
19. S. Hu, L. Zhang, and N.J. Dovichi. Characterization of interaction between phospholipids and proteins by capillary electrophoresis with laser induced fluorescence detection. *J. Chromatogr. A.* **934:** 369–375 (2000).
20. A.A. Garcia. *Bioseparation Process Science*. Blackwell Publishing, London, UK, 1999.
21. I. Elfman-Börjesson and M. Härröd. Analysis of non-polar lipids by HPLC on a diol column. *J. High Resolut. Chromatgr.* **20(9):** 516–518 (2005).
22. S. Perona Javier and V. Ruiz-Gutierrez. Quantification of major lipid classes in human triacylglycerol-rich lipoproteins by high-performance liquid chromatography with evaporative light-scattering detection. *J. Sep. Sci.* **27(9):** 653–59 (2004).
23. Key Benefits and Features of Inertsil Diol—Part of the Advanced Inertsil 3-Series of Phases. Copyright 2002-2007 GL Sciences, Inc.

Manuscript received July 3, 2008;
revision received August 21, 2008.