

# HPLC with Flame Ionization Detection: Class Separation, Linearity of Response, and Quantification of Sterols, Glycerides, and Phospholipids

---

**ROBERT J. MAXWELL,\* EDWIN H. NUNGESEER, WILLIAM N. MARMER, and THOMAS A. FOGLIA**  
*U.S. Department of Agriculture, ARS, Eastern Regional Research Center,  
600 East Mermaid Lane, Philadelphia, Pennsylvania 19118*

Reprinted from

**LCGC**

VOLUME 5 NUMBER 9

MAGAZINE OF  
LIQUID AND GAS CHROMATOGRAPHY

# HPLC with Flame Ionization Detection: Class Separation, Linearity of Response, and Quantification of Sterols, Glycerides, and Phospholipids

---

**A recently introduced HPLC flame ionization detector was evaluated for its suitability as a mass-sensitive detector for quantifying lipids. Representative sterol esters, sterols, triglycerides, and glycerophosphatides were individually analyzed in replicate determinations. Each of the lipid classes give a linear response over the tested range of 6–200  $\mu\text{g}$ . Response factors varied with lipid class. Mixtures of these lipid classes were analyzed on a silica column treated with ammonium hydroxide to give complete, reproducible class separation of all components.**

---

## INTRODUCTION

The simultaneous separation and quantitation of lipid classes by high performance liquid chromatography (HPLC) had, until recently, eluded chromatographers. Most purported lipid separations were accomplished using ultraviolet (UV) or refractive index (RI) detection with a variety of column types and solvent systems (1,2). Use of such systems with UV detection often causes several unresolvable problems: the only functional groups detectable by UV are the carbonyl groups and double bonds contained in the fatty acid chains, which absorb in the 200–215 nm region. Because this absorbance is dependent on the concentration and degree of unsaturation of the molecular species, direct quantitation of a complex lipid mixture is not possible with UV detection (3). Furthermore, the most effective solvent systems developed for the TLC analysis of lipids cannot be used with UV detection because they are not transparent in the region where lipids absorb; with refractive index detection, lipid class separa-

tions are not possible because gradients are necessary for elution of individual lipid classes.

Difficulties with the HPLC detection of lipids have led to renewed interest in other types of detection, such as light scattering (4,5) or flame ionization, that do not have the inherent problems of UV and RI detectors — although the linearity and response of the light-scattering detector may be influenced by the nature of the eluting solvent (5). In 1981, Phillips and Privett reported the separation and quantitation of a lipid mixture by normal-phase HPLC with flame ionization detection (FID) (6). Their mass-sensitive detector, constructed in the laboratory, used an endless moving metal belt to convey the column eluate to the flame ionization detector or mass spectrometer after evaporation of the mobile phase (7). More recently, a flame ionization detector for HPLC that uses a somewhat different method for eluate transport was made commercially available by Tracor Instruments (Austin, Texas). The column eluate is applied through a jet to a heated circular quartz belt mounted on a revolving metal disk. The solvent is removed through a vacuum port; sample remaining on the belt passes through the detector flame. The belt then passes through a second, hotter cleaning flame before additional eluate is deposited on the belt.

We have evaluated the Tracor flame ionization detector for its suitability as a mass-sensitive detector in the analysis of lipid mixtures by normal-phase HPLC. Two reports have recently described the use of the same detector for the separation of lipid molecular species by reversed-phase HPLC (8,9); however, linearity studies of individual lipid classes such as sterols, glycerides, and glycerophosphatides have not been reported. In our investigation, the individual lipid classes first were tested for their linearity of response to the detector. A gradient system was then developed for the separation and analysis of complex mixtures using lipid standards as a model system.

## EXPERIMENTAL

**Standards and solvents:** Cholesteryl oleate and cholesterol (Nu-Chek-Prep, Elysian, Minnesota), triolein (Nippon Oil & Fat Co., Amagasaki, Japan), and dipalmitoylphosphatidylcholine and dilauroylphosphatidylcholine (Sigma Chemical Co., St. Louis, Missouri) were of the highest purity available and were used without further purification. Solvents (Burdick & Jackson, Muskegon, Michigan) were used as received except acetonitrile, which caused excessive baseline noise until it was distilled from phosphorus pentoxide.

**Apparatus:** Two separate systems were used in these experiments. For linearity determinations, the HPLC system consisted of two model 110A pumps, a model 421 system controller, and an Altex 210 injector with a 20- $\mu\text{L}$  loop (all from Beckman Instruments, San Ramon, California). The data were collected on a model 3390A reporting integrator (Hewlett-Packard, Palo Alto, California). Analyses were performed on a 25 cm  $\times$  4.6 mm, 10- $\mu\text{m}$  EM Lichrosphere Si-100 column (Bodman Chemicals, Media, Pennsylvania) (column A). Subsequently, we found that the lipid class separations required a ternary gradient; therefore we used a series 400 liquid chromatograph with quaternary pumping capability (Perkin-Elmer, Norwalk, Connecticut) that had a model 7125 injection valve (Rheodyne, Cotati, California) with a 6- $\mu\text{L}$  loop. The column used in the gradient analyses was prepared (by ES Industries, Marlton, New Jersey) as follows: Spherisorb S-GP, 8  $\mu\text{m}$  (Phase Separations, Norwalk, Connecticut) was mixed with concentrated NaOH and allowed to sit for 48 h. The Spherisorb material was filtered, dried, and packed into a 30 cm  $\times$  2.1 mm column (column B). A 5 cm  $\times$  2.1 mm guard column containing the same material was also used (6).

**Standards:** For linearity studies, various concentrations of each of the five substrates were prepared by serial dilution of gravimetrically measured amounts of each compound. For each substrate, six repeated injections at each concentration were made onto column A.

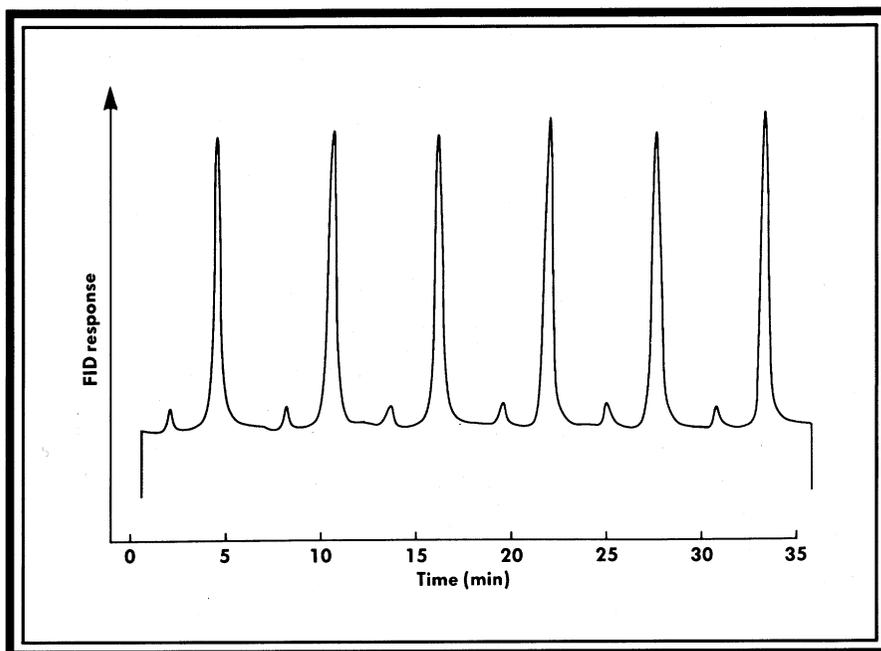
## RESULTS AND DISCUSSION

To assess the response of the flame ionization detector to lipids having a broad range of structures, functionality, chemical charge, molecular weights, and volatility, five representative compounds were chosen for study. Three were neutral lipids: a sterol (S) (cholesterol), a steryl ester (SE) (cholesteryl oleate), and a triglyceride (TG) (triolein). Two were polar lipids: a phosphatidylethanolamine (PE) (dilauroyl PE), and a phosphatidylcholine (PC) (dipalmitoyl PC). These compounds represent the classes most often encountered in routine lipid analysis. Other minor classes such as phosphatidylserines or phosphatidylinositols would be expected to respond similarly to those classes most similar in structure and retention times.

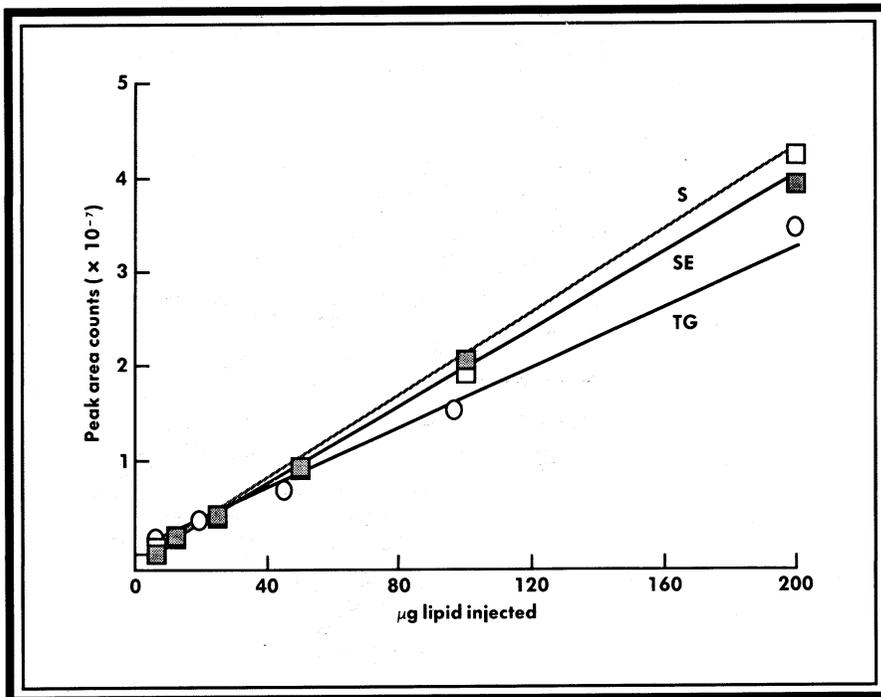
Initial studies developed the optimum conditions for elution of individual lipids under normal-phase conditions to assess relative responses. In contrast to the limitations in solvent choice associated with UV detectors when lipids are analyzed, the flame ionization detector allows a much wider variety of solvents to be used. Nevertheless, there are some restrictions. For instance, acetic acid, *n*-propanol, and toluene, among others, should not be used because their boiling points are too high for efficient evaporation. Buffers that leave residues following evaporation give reduced detector response, but ammonium buffers are acceptable. Within these limits, most of the solvents ordinarily used for lipid analyses by column chromatography or TLC may be used.

High baseline noise for replicate injections of very dilute samples was noted in early experiments using reporting integrators other than the HP 3390A. Those problems persisted when parameters such as peak threshold, peak width, response time, and minimum detectable peak areas were adjusted. The HP 3390A gave relatively flat baselines and reproducible results from replicate injections. Newer models of the Tracor 945 flame ionization detector are reported to have an enhanced ability to filter electronic noise and therefore may be compatible with other reporting integrators.

In experiments designed to test the linear range of this detector, individual lipids were prepared as solutions starting at 200  $\mu\text{g}/20 \mu\text{L}$  that were serially diluted until the compound showed no detectable response. We conducted repeatability studies by injecting the sample six times at each concentration; a representative example from these studies appears in Figure 1 for dipalmitoylphosphatidylcholine. Peak shapes were symmetrical and peak areas for each injection were repeatable; the coefficient of variation within each set



**FIGURE 1:** Repeatability of detector response for six injections of dipalmitoylphosphatidylcholine. CV = 2.2%. Column: A (see text); mobile phase: 95:95:20 (v/v) chloroform/methanol/water; flow rate: 1.0 mL/min; sample size: 100  $\mu\text{g}$  per 20- $\mu\text{L}$  injection.



**FIGURE 2:** FID response for neutral lipids. S = cholesterol ( $r = 0.9990$ ); SE = cholesteryl oleate ( $r = 0.9992$ ); TG = triolein ( $r = 0.9979$ ). Column: A; mobile phase: 1:1 (v/v) acetonitrile/dichloromethane; flow rate: 1.0 mL/min.

was  $\sim 2.2\%$ . Similar chromatograms were obtained at the other tested concentrations cited and for all of the other pure standard lipids tested.

The FID response of the individual standards over the tested range of concentration is shown in Figure 2 for the three neutral lipids and in Figure 3 for the two polar lipids. Although slight variations in the slopes for indi-

vidual standards were observed, the response for each compound tested was linear over the range of 6–200  $\mu\text{g}$ , with correlation coefficients ( $r$ )  $> 0.9978$ . The elution solvent for the three nonpolar standards (Figure 2) was a 1:1 (v/v) mixture of acetonitrile/dichloromethane, whereas the polar standards (Fig-

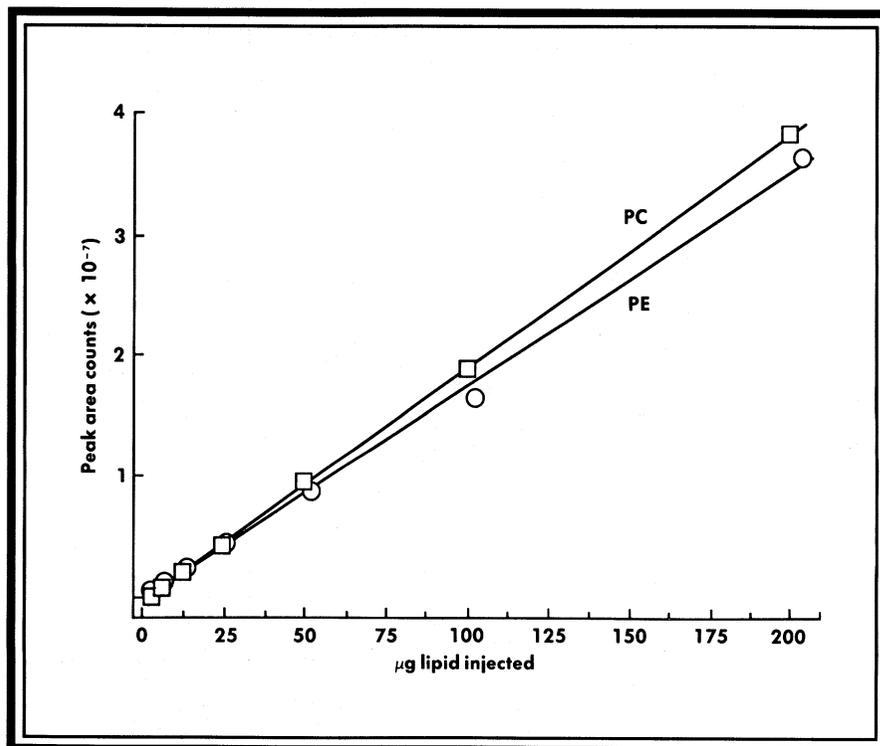
ure 3) were eluted with a 95:95:20 (v/v) mixture of chloroform/methanol/water. Nevertheless, similar peak area results were obtained for both sets, indicating that linearity of response was not affected by changes in mobile phase composition or polarity.

The linearity studies were carried out using isocratic conditions on column A. Because of the complexity of a typical lipid mixture, however, gradient conditions are required to effect complete separations of individual classes. Initially, we attempted to separate mixtures of polar and nonpolar lipids using binary gradient systems. Several combinations were employed using solvent systems normally used in lipid TLC analysis. Problems with reproducibility were encountered with every system that was tested, however. Therefore, a ternary gradient first proposed by Phillips and Privett (6) for their laboratory-made flame ionization detector was tried and found to be successful for the quantitative, reproducible separation of lipid mixtures. This method requires that the stationary phase silica be treated with concentrated  $\text{NH}_4\text{OH}$  before packing, a technique that conditions the packing and allows reproducible gradient analyses. The packed column then must be equilibrated using the gradient shown in Figure 4a. To ensure column reproducibility, the entire specified gradient must be completed after all of the lipids in the profile have eluted. The mobile phase contains  $\text{NH}_4\text{OH}$ , which caused only minor deterioration of the flame ionization detector's quartz belt — so slight that it did not result in undue baseline noise. A chromatogram of the pure standards (Figure 4b) shows that a slight baseline drift occurred during the introduction of  $\text{NH}_4\text{OH}$  into the mobile phase; this effect was reproducible, however, and did not affect peak shapes or retention times. The peak asymmetry factors for this column were high but were within the acceptable range (1–2) for computing apparent  $N$  values (10). Dipalmitoylphosphatidylcholine was eluted in <35 min, a result that compares favorably with reported separations that used UV (2) and light-scattering mass detectors (4,5).

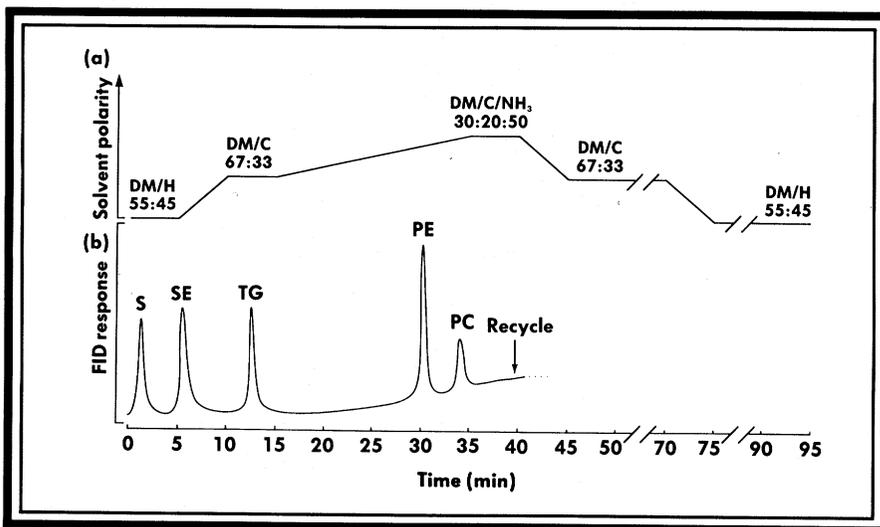
The flame ionization detector thus allows for the direct separation and quantitation of the major lipid classes without the problems encountered with other detector types. Recent results by other researchers in this laboratory have shown that gradient HPLC-FID allows quantitation of another lipid class, cholesterol oxides, and that compounds of this class show linear responses at levels even below those determined in the present study (11).

## REFERENCES

- (1) F.B. Jungalwala, J.E. Evans, and R.H. McCluer, *J. Biochem.* **155**, 55 (1976).
- (2) G.M. Patton, J.M. Fasulo, and S.J. Robins, *J. Lipid Res.* **23**, 190 (1982).
- (3) W.W. Christie, *Z. Lebensm.-Unters. Forsch.* **181**, 171 (1985).



**FIGURE 3:** FID response for polar lipids. PC = dipalmitoylphosphatidylcholine ( $r = 0.9999$ ); PE = dilauroylphosphatidylethanolamine ( $r = 0.99991$ ). Column: A; mobile phase and flow rate same as in Figure 1.



**FIGURE 4:** Class separations of pure lipid standards using gradient elution. (a) Gradient conditions. DM =  $\text{CH}_2\text{Cl}_2$ ; H = hexane; C =  $\text{CHCl}_3$ ;  $\text{NH}_3$  = 6% concentrated  $\text{NH}_4\text{OH}$  in methanol. (b) Chromatogram. Column: B; sample size range: 156–192  $\mu\text{g}$ ; injection volume: 6  $\mu\text{L}$ . Peaks: S = sterol (asymmetry factor [ $A_s$ ] = 1.20); SE = steryl ester ( $A_s$  = 1.16); TG = triglyceride ( $A_s$  = 1.20); PE = phosphatidylethanolamine ( $A_s$  = 0.75); PC = phosphatidylcholine ( $A_s$  = 1.20).

- (4) W.W. Christie, *J. Lipid Res.* **26**, 507 (1985).
- (5) J.L. Robinson and R. Macrae, *J. Chromatogr.* **303**, 386 (1984).
- (6) F.C. Phillips and O.S. Privett, *J. Am. Oil Chemists' Soc.* **58**, 590 (1981).
- (7) O.S. Privett and W.L. Erdahl, *Anal. Biochem.* **84**, 449 (1978).
- (8) L.A. Smith, H.A. Norman, S.H. Cho, and G.A. Thompson, Jr., *J. Chromatogr.* **346**, 291 (1985).
- (9) H.A. Norman and J.B. St. John, *J. Lipid Res.* **27**, 1104 (1986).
- (10) L.R. Snyder and J.J. Kirkland, *Introduction to Modern Liquid Chromatography*, 2nd ed. (Wiley-Interscience, New York, 1979), p. 222.
- (11) G. Maerker, E. Nungesser, and M. Zulak, *J. Agric. Food Chem.*, in press. ■