paper on methodology

Separation and identification of triglycerides, cholesteryl esters, cholesterol, 7-dehydrocholesterol, dolichol, ubiquinone, α-tocopherol, and retinol by high performance liquid chromatography with a diode array detector

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Abstract A simple isocratic high performance liquid chromatography (HPLC) system is described that allows separation and identification of cholesteryl esters, triglycerides, ubiquinone, α-tocopherol, dolichol, cholesterol, 7-dehydrocholesterol, and retinol. This consisted of a normal phase cyanopropyl column with 0.1% isopropanol in heptane as the solvent. The effluent was monitored with an LKB model 2140 diode array detector which enabled the lipids to be identified by their characteristic absorption spectra. This system was applied to a sample of dog liver in which cholesteryl esters, retinyl esters, triglycerides, ubiquinone, dolichol, cholesterol, and retinol were identified. Retinyl esters and vitamin D esters were identified by their similarity in absorption spectra to retinol and vitamin D. A system to transfer and store the chromatograms on the VAX PDP-11 or an optical disc is also described. —**Greenspan, M. D., C-Y. L. Lo, D. P. Hanf, and J. B. Yudkovitz.** Separation and identification of triglycerides, cholesteryl esters, cholesterol, 7-dehydrocholesterol, dolichol, ubiquinone, α-tocopherol, and retinol by high performance liquid chromatography with a diode array detector. *J. Lipid Res.* 1988. **29:** 971–976.

Supplementary key words dog liver lipids • lipid-soluble vitamins

HPLC separation of lipids has been carried out with both normal and reverse phase chromatography. In general the reverse phase systems have been used to separate molecular species of the various lipids (1–7), while normal phase chromatography is used for separation of various classes of lipids (8–12). We have been interested in examining the lipids and lipid-soluble vitamins in dog liver and have utilized a normal phase system to identify and quantitate the lipid classes present. However, because of the large diversity of lipids (ranging in polarity from phospholipids and gangliosides to cholesteryl esters and triglycerides), it has not been possible to separate all classes of lipids in a single chromatographic run. Thus, in a polar system where phospholipids would separate, neutral lipids would not chromatograph, and in a nonpolar system where cholesterol separated from triglyceride, the phospholipids would probably not be soluble. Therefore, a balance had to be struck in finding a chromatography system that would separate and identify as many lipids as possible.

Two articles dealing with the separation and identification of these lipids and lipid-soluble vitamins have recently come to our attention. Palmer, Anderson, and Jolly (12) described a normal phase system that separated ubiquinone, cholesterol, and dolichol after removal of the phospholipids, and Miller and Yang (7) utilized a diode array detector to identify retinol, tocopherol, and various carotenoids in plasma. By taking advantage of the different ultraviolet absorption spectra of the compounds, Miller and Yang (7) were able to identify and determine the purity of the samples as they eluted from a reverse phase column.

By combining the chromatographic system of Palmer et al. (12) with the spectral analysis detection as described by Miller and Yang (7) we have been able to separate and identify cholesteryl esters, triglycerides, ubiquinone, α-tocopherol, dolichol, cholesterol, 7-dehydrocholesterol, and retinol. Retinyl esters and vitamin D esters were also

Abbreviations: HPLC, high performance liquid chromatography.

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identified by their characteristic ultraviolet spectra, but we could not be certain of their identification because of the unavailability of standards.

MATERIALS AND METHODS

Materials

Ubiquinone, α-tocopherol, dolichol, 7-dehydrocholesterol, and retinol standards were purchased from Sigma Chemical Co.; cholesterol and cholesteryl oleate were from Kodak Chemical Co.; triolein was from Nu-Chek-Prep. All solvents were HPLC grade and filtered through Millipore 0.22-μm membranes prior to use. Silica gel columns with a stainless-steel frit were Bond Elut SI #601313 from Analytichem International. These were washed before use with 5 ml each of diethyl ether and methanol.

HPLC equipment

The HPLC column used throughout these studies was a 6-micron, 0.46 × 25 cm, Zorbax CN column (Du Pont). Knauer #64 pumps operated with a Beckman 421 controller were the chromatographic equipment, and a Waters Wisp 710B was used for injection of the sample onto the column. Detection was with an LKB 2140 Diode Array Detector. The instrument was set to scan the column effluent between 190 and 370 nm (1 nm resolution). In addition, the detector has four analog outputs that were set to 205, 272, 296, and 325 nm and connected to a Nelson analytical 4416 data system. The integration of the various fractions was carried out in the Nelson system at these wavelengths. Standard curves were prepared by chromatographing known amounts of triolein, cholesteryl oleate, ubiquinone, α-tocopherol, dolichol, cholesterol, 7-dehydrocholesterol, and retinol. **Fig. 1** describes the absorption (calculated as area units under the peaks × 10^6) for the different lipids. The particular wavelength at which the absorption was measured is documented in the legend to Fig. 1. Dolichol, α-tocopherol, ubiquinone, and 7-dehydrocholesterol could be analyzed in the range of 1–25 μg (Fig. 1a), retinol at the level of 0.5–10 μg (Fig. 1b), and cholesterol, cholesteryl oleate, and triolein from 5–100 μg (Fig. 1c). Both ubiquinone and α-tocopherol have greater extinction coefficients at 200 than at 272 and 296 nm, respectively, and the sensitivity of these compounds could be increased when necessary by monitoring them at 200 nm. The Nelson software allowed the appropriate curves for each of the lipids to be stored and the unknowns were compared to the standard curves in the computer with the readout being both micrograms and area units.

Data handling

For the analysis of the samples, complete ultraviolet spectra (from 190 to 370 nm) were taken every 0.2 sec and the spectra were integrated at 4-sec intervals with the LKB 2140 Diode Array Detector system. This yielded ex-

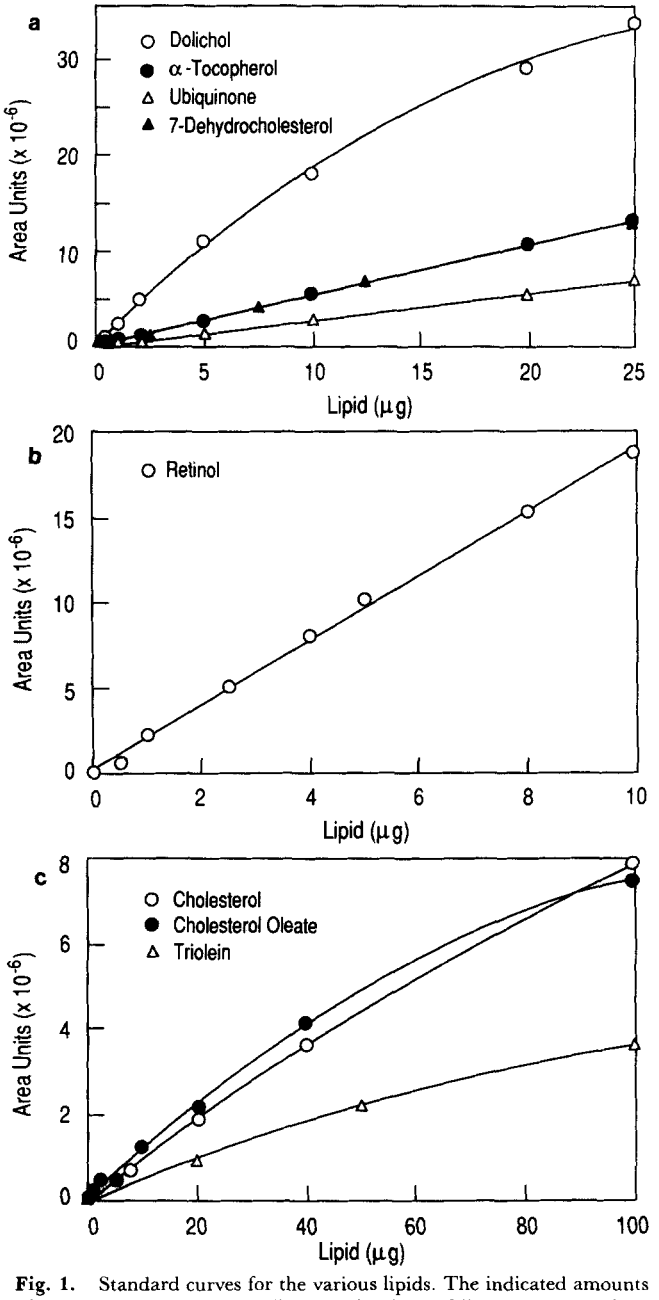

Fig. 1. Standard curves for the various lipids. The indicated amounts of the various compounds dissolved in the mobile phase were chromatographed as outlined in the Methods section. In Fig. 1a, dolichol (○) was measured at 205 nm, ubiquinone (△) and 7-dehydrocholesterol (▲) at 272, and α-tocopherol (●) at 296 nm; retinol in Fig. 1b was measured at 325 nm; and in Fig. 1c cholesterol (○), cholesterol oleate (●), and triolein (△) were measured at 205 nm. The standard curves were stored in the computer as described in the text.

tremely large data files for our standard 25-min run length (approximately 0.152 megabytes per file). As it is extremely inconvenient to store large numbers of these files on standard floppy disks or even on a Winchester Drive, it was decided to store the data on a VAX computer. The major problem encountered with the transfer of these files was that they were a combination of binary and ASCII codes and most communication protocols were designed for either one or the other, but not both. Kermit, a protocol written at the Columbia University Center for Computing Activities, was designed to handle all types of files and has the added advantage of being an error-free file transfer protocol. We therefore used Kermit to transfer the files to the VAX for permanent storage. As the number of files generated in these studies increased, the amount of time necessary to transfer the files to the VAX, even at 4800 Baud, became extremely long. Therefore, a system was instituted to archive data on a Franklin Telecom Optical Disk subsystem. This allowed storage of data almost as rapidly as storage on a magnetic disk drive. In addition, we have approximately 1400 data files randomly accessible on each side of the 220 Mbyte optical disk.

Extraction and chromatography of lipids

Lipids were extracted from the liver by the Bligh-Dyer method (13). One gram of liver was added to 3 ml of methanol–chloroform 2:1, the mixture was homogenized with a Polytron homogenizer, and shaken intermittently for 1 hr. After centrifugation for 5 min at ca. 1000 *g*, the clear supernatant was removed. Another 3 ml of the methanol–chloroform plus 0.8 ml of saturated NaCl was added to the residue and the extraction process was repeated. The supernatants were combined and 2 ml each of chloroform and saturated NaCl were added which resulted in a phase separation. This mixture was vortexed, centrifuged, and the chloroform layer was removed and concentrated to dryness under N_2. The residue was dissolved in 0.5 ml of chloroform and added to a washed Bond Elut column and the lipids were eluted with 5 ml of diethyl ether. The diethyl ether fraction was taken to dryness and the residue was dissolved in 0.4 ml of the mobile phase (0.1% isopropanol in heptane). Phospholipids and other polar lipids could be eluted from the silicic acid column with 5 ml of methanol.

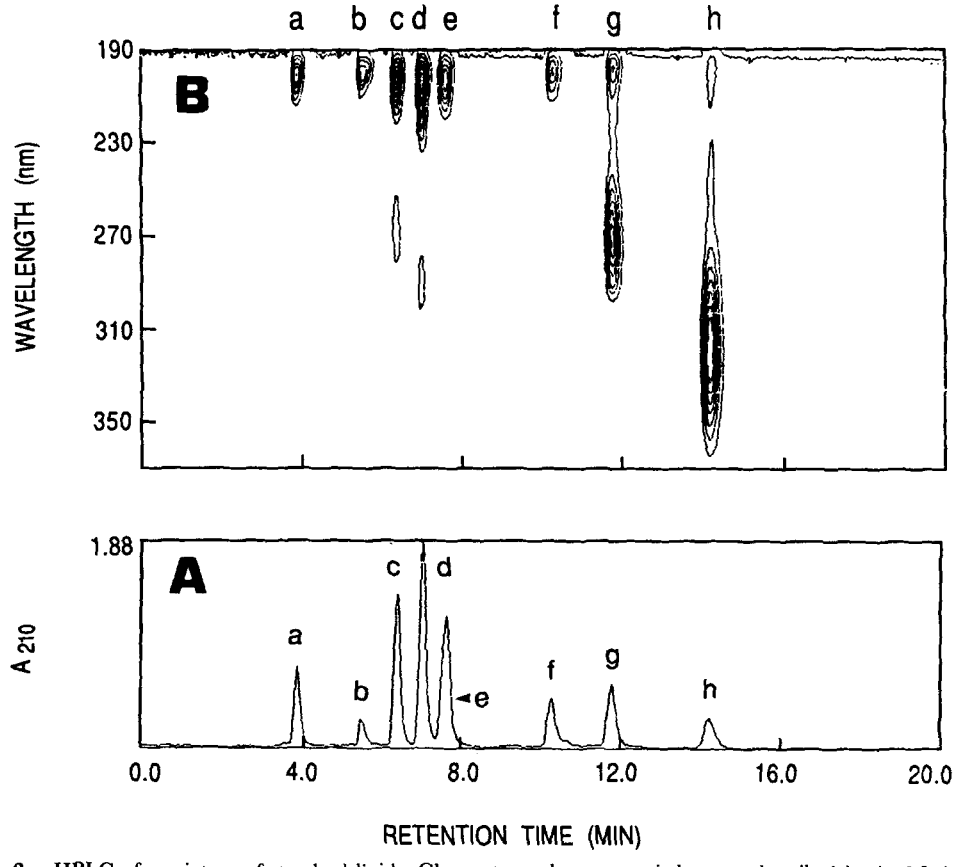

Fig. 2. HPLC of a mixture of standard lipids. Chromatography was carried out as described in the Methods section. Fig. 2a is a chromatogram of the lipids recorded at 210 nm, while Fig. 2b is an isogram of the same sample scanned and recorded between 190 and 370 nm. The fractions shown correspond to 50 μg of cholesteryl oleate (a), 100 μg of triolein (b), 5 μg of ubiquinone (c), 5 μg of α-tocopherol (d), 5 μg of dolichol (e), 50 μg of cholesterol (f), 25 μg of 7-dehydrocholesterol (g), and 10 μg of retinol (h).

An aliquot of the lipid extract was injected onto the cyanopropyl column and the lipids were eluted isocratically with 0.1% isopropanol in heptane at 1 ml/min at room temperature.

RESULTS

A chromatogram of a variety of standard lipid and lipid-soluble vitamins in the normal phase system with the cyanopropyl column is shown in **Fig. 2.** The results of this are described in the conventional chromatogram recorded at 210 nm shown in Fig. 2a. Baseline separations were seen with all of the lipids and each compound chromatographed with the same retention time as a previously chromatographed individual standard. Further confirmation of the identity of these lipids is shown with the ultraviolet spectra of the corresponding isogram recorded between 190 and 370 nm in Fig. 2b. Cholesteryl oleate (a), triolein (b), dolichol (e), and cholesterol (f) had uncomplicated spectra with a single peak around 200 nm. Ubiquinone (c) with a retention time of 6.8 min showed major absorption bands at 200 and 272 nm, while α-tocopherol (d) had peaks at 200 and 296 nm with a shoulder at 234 nm. 7-Dehydrocholesterol (g) showed an absorbance maximum at 200 nm and a double peak at 272 and 276 nm followed by retinol (h) eluting at 15.5 min with a characteristic absorption maximum at 325 nm and a small peak at 200 nm.

A sample of dog liver was extracted and chromatographed as described in the Methods section. The chromatogram at the 210 nm wavelength is seen in **Fig. 3a,** and the isogram is recorded between 190 and 370 nm in Fig. 3b. The fraction with the shortest retention time (fraction a) was complicated and obviously composed of more than one compound. Other fractions chromatographed with retention times and spectra corresponding to that of triolein (b), ubiquinone (c), dolichol (e), cholesterol (f), and retinol (h). Two unknown fractions were seen (retention times of 6.95 and 9.26 min, respectively) which did not show unique spectra other than absorption around 200 nm and were not further identified. The identity of the components of the fraction nearest the solvent front was ascertained by examining the ultraviolet absorption spectra in detail. ***Fig. 4*** *shows three spectra from different* portions of fraction a. These spectra were extracted from the isogram. The retention time of the leading fraction at

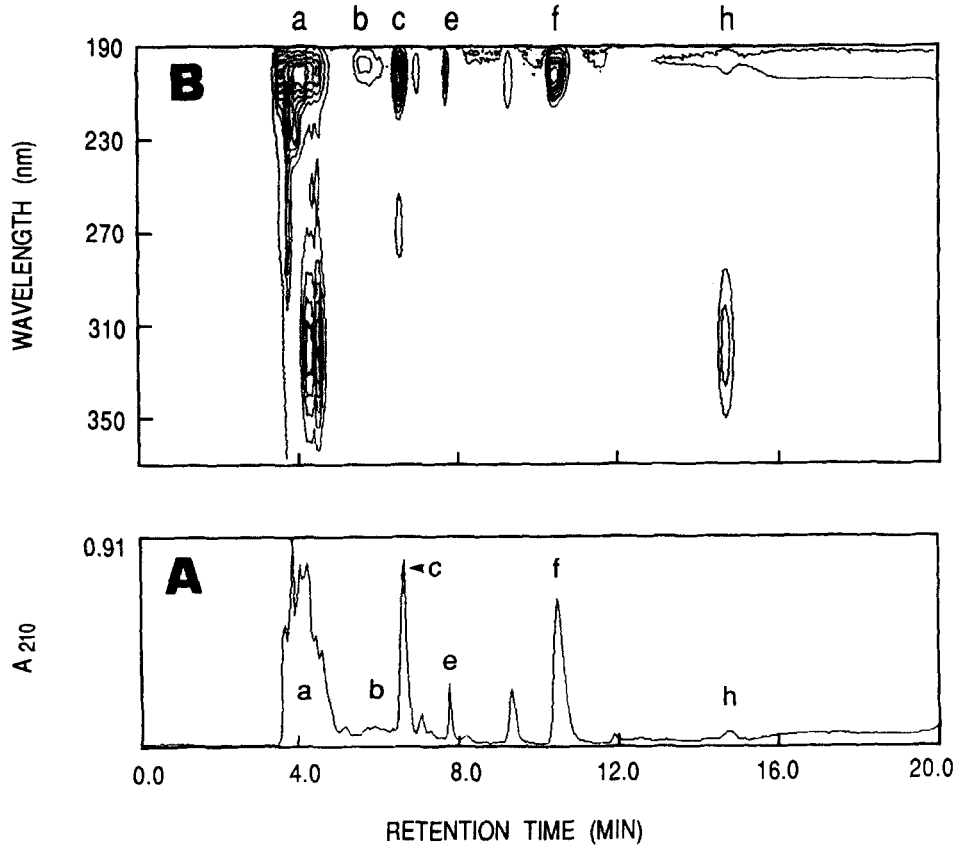

Fig. 3. HPLC of a lipid extract from dog liver. Fig. 3a is a chromatogram of the dog liver lipids at 210 nm and Fig. 3b is the isogram scanned between 190 and 370 nm. The peaks identified correspond to those of Fig. 2.

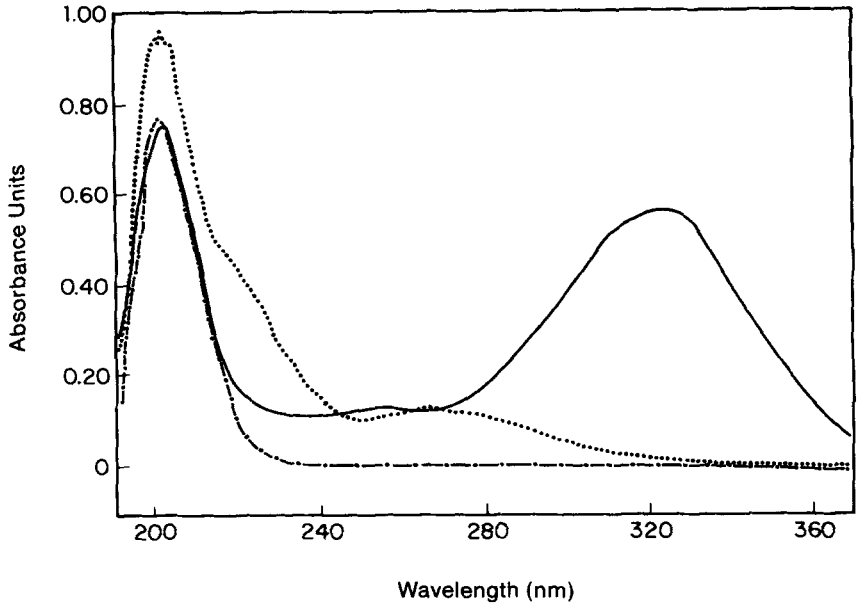

Fig. 4. Ultraviolet absorption spectra of three components from fraction a, Fig. 3. The UV spectra of fractions with the retention times of 3.58 min (-●-●), 3.75 min (●●●●●), and 4.34 min (——) were separated and recorded by the LKB 2140 diode array detector.

3.58 min suggested that it was cholesteryl esters, which was indicated by the simple spectrum with a single band around 200 nm. The small peak at 4.34 min had the characteristic 325 nm absorption of retinol. Thus the early retention time, the absorption at 325 nm, and the increase in the ratio of 201/325 nm over that of the retinol standard (data not shown) were further indications that this fraction was probably composed of retinyl esters. Confirmation of this was seen on disappearance of this peak and increase in the free retinol after saponification of the sample (data not shown). The spectrum of the largest peak at 3.75 min showed the maximum at 201 nm, a shoulder at 213 nm, and another broad band at 265 nm. The absorption band at 265 nm suggests that this fraction was probably a vitamin D ester. Further characterization was not carried out.

A study was also carried out to determine the recovery of each sample. One hundred μg of each lipid was dissolved in 0.5 ml of chloroform and added to the silicic acid column and eluted as described in the legend to **Table 1.** The diethyl ether eluate was dried and redissolved in the mobile phase and an aliquot was chromatographed by HPLC. Hydrolysis of the lipids was carried out by addition of ethanolic KOH to the dried diethyl ether fraction, followed by heating, solvent extraction, and chromatography as described in the legend to Table 1. The recovery was over 90% for ubiquinone, cholesterol, and dolichol, and slightly less (88%) for α-tocopherol. Saponification did not affect the recovery of dolichol and cholesterol, whereas all of the ubiquinone, half of the α-tocopherol, as well as the triglycerides and most of fraction a (from Fig. 3) were lost by the base treatment.

DISCUSSION

The studies presented in this paper demonstrate a simple method for identification of a variety of lipids and lipid-like vitamins in an isocratic system. The basic chromatographic system itself has already been described by Palmer et al. (12), and we have simply extended this by combining it with a diode array detector to characterize each fraction. The power of this system was realized when we were able to characterize the early eluting peaks as cholesteryl esters and most likely retinol and vitamin D esters. These compounds were identified by virtue of their characteristic ultraviolet absorption spectra. Being able to identify fractions at various wavelengths has the obvious

TABLE 1. Recovery of lipids after extraction, hydrolysis, and HPLC

Lipid	Percent Recovery	
	Bligh-Dyer Procedure[a]	Hydrolysis Procedure[b]
Dolichol	94%	98%
Cholesterol	96	96
Ubiquinone	92	11
α-Tocopherol	88	55

[a] As described in the Methods section of this paper.
[b] One ml of 10 N KOH and 1.0 ml of ethanol were added to a 1.5-ml solution of the standards containing 25% ascorbic acid. This solution was heated to 70°C for 30 min and then extracted twice with 4 ml of hexane. The hexane was dried and the residue was dissolved in 400 μl of 0.1% isopropanol in heptane and chromatographed as described in the Methods section.

advantage of enabling integration of two closely chromatographing fractions where large amounts of one might obscure the other at a single wavelength. Thus, early in this study, plasticizers eluted inadvertently from the prepacked silicic acid column interfered with ubiquinone and α-tocopherol at 205 nm. However, the data were not lost because the ubiquinone and tocopherol were also measured at 272 and 296 nm, respectively, where these plasticizers did not absorb. A similar problem was also occasionally encountered when large amounts of triglycerides were present in samples and caused interferences with the ubiquinone at 205 nm.

The four analog outputs of the detector made it possible to integrate each chromatogram at 205, 272, 296, and 325 nm simultaneously on the Nelson Analytical 4416 system. Standard curves were established and stored for triglycerides, cholesterol, cholesteryl esters, and dolichol at 205 nm, for ubiquinone and 7-dehydrocholesterol at 272 nm, for α-tocopherol at 296 nm, and for retinol at 325 nm. This allowed direct readings in micrograms from the computer for each of these fractions from the dog liver. An additional advantage of the diode array detection system, which was utilized in these studies but is not reported here, is the determination of purity of a fraction by examination of the constancy of the spectra across a peak or the ratio of the absorbances at two wavelengths across a peak.

The use of the silicic acid columns to remove phospholipids prior to HPLC is also recommended. This extends the life of the column since the polar lipids are not soluble in the isopropanol–heptane system.

We would also like to point out that heptane was used instead of hexane as noted by Palmer et al. (12). When hexane was used as the chromatography solvent, changes were noted in the retention times of the lipids as the day progressed. This was probably related to a loss of hexane from the solvent mixture by evaporation, resulting in an increase in the isopropanol concentration. This change was especially noticeable on warm days. Heptane, with a boiling point of 98°C obviated this problem. With the heptane–isopropanol system, we found that a supply of solvent could be used a number of days with no significant change observed in the chromatography.

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