Adaptation of an HPLC System to Quantify Cholesterol

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An HPLC system for separating oxidized components of crude soybean oil was adapted for use in quantifying cholesterol. At 210 nm there was linear integrator repsonse to concentration of cholesterol. The response was linear at four detector ranges, and the relationship remained linear when data were adjusted to one range. Spray-dried egg yolks contained 3,048 mg (± 117) of cholesterol per 100 g, a value similar to literature measures.

Cholesterol is a lipid constituent found in animal products. Much attention has been focused on levels of cholesterol in different foods. Current methodology for the analysis of cholesterol often is time consuming, and many techniques that have been developed are used to observe oxidized forms of cholesterol rather than to quantify cholesterol.

Recent high pressure liquid chromatographic (HPLC) techniques have used non-aqueous reversed-phase systems with saponified or esterified derivatives to determine cholesterol in foods (1-3). The mobile phases in these reverse phase systems range from nonpolar (1) [1/99.9 (2propanol/hexane)] to extremely polar, (2) [methanol] and UV detection was at 205 nm, 230 nm and 254 nm.

Other researchers have used "normal phase" silica to observe oxygenated cholesterols and related compounds (3-6). For analysis on normal silica, cholesterol does not have to be derivatized. Detection has been achieved by refractive index as well as UV. Solvent systems vary in the percentage of 2-propanol (IPA) in hexane (4-7). These solvents can elute numerous autoxidation products of cholesterol, and the greater the degree of oxidation, the longer the retention times. Tsai and Hudson (6) removed the triglycerides and cholesterol from egg yolk lipids while estimating cholesterol oxide.

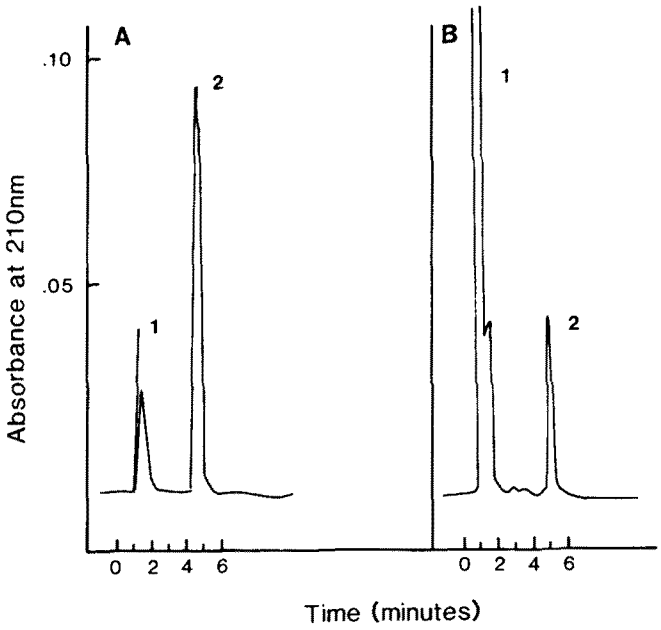

FIG. 1. HPLC separation at 210 nm of (a) purified cholesterol in 25% IPA in hexane and (b) egg oil. Eluting solvent was 0.75% IPA in hexane; 2 ml/min; column, ultrasphere silica (15 cm × 4.6 μm).

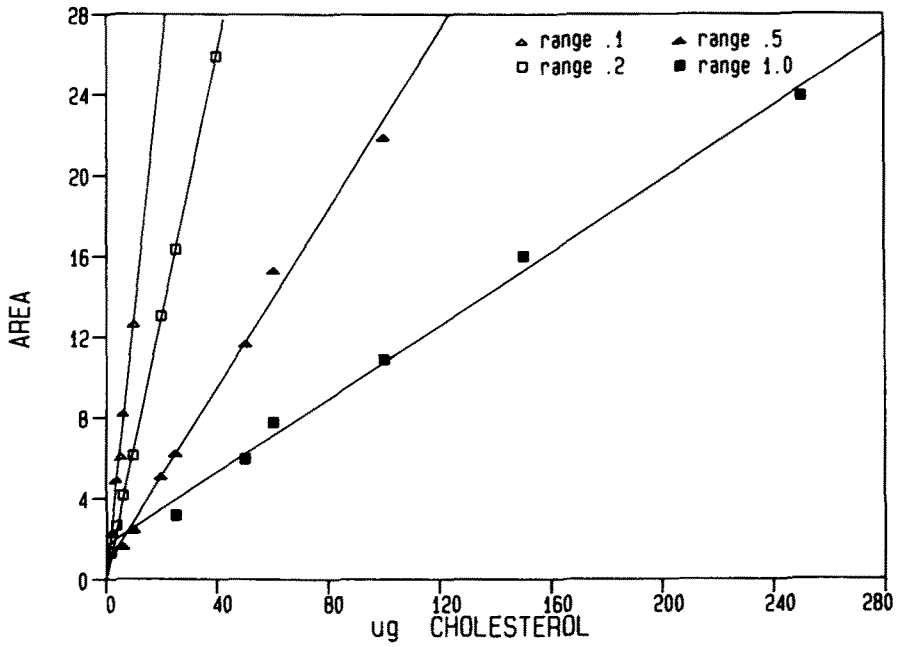

FIG. 2. HPLC calibration curves for cholesterol at four ranges of the Hitachi variable wavelength spectrophotometer.

In this laboratory a technique was developed for separation of unoxidized triglycerides from oxidation products of soybean oil using 0.75% IPA/hexane (8). Unoxidized triglycerides were eluted first, and conjugated diene compounds (i.e. oxidation products) had longer retention volumes. Our current research involves extracting cholesterol from spray-dried egg yolks and developing a low-fat, low-cholesterol egg product. Therefore, this research was undertaken to develop a technique for rapidly assessing the amount of cholesterol in the fat of full-fat and defatted spray-dried egg yolks using HPLC.

MATERIALS AND METHODS

The liquid chromatograph used consisted of a Bechman Model 322 with Hitachi (Model 155-99) variable wavelength spectrophotometer set at 210 nm and a Varian CDS 111 electronic integrator. The HPLC column was a 5 mm × 15 cm Ultrasphere Si (Beckman) packed with 5μm spherical particle silica. A guard column packed with Pellosil (Whatman, 30-38μm glass bead with pellicular silica) was used to increase the life of the column. The mobile phase was 0.75% IPA in hexane flowing at 2.0 ml/min.

Cholesterol and stigmasterol standards were obtained from Nuchek Prep (Elysian, Minnesota) and Sigma Chemical Co., (St. Louis, Missouri), respectively, and dissolved in IPA/hexane mixture. Cholesterol concentrations ranged from 0.1 μg/μl to 5 μl, and on-column sample size was adjusted from 2 μg to 250 μg using different size injection loops. Standard curves were determined at various ranges on a 0-1.0 absorbance scale. The amount of cholesterol in spray-dried egg yolk was determined by extracting the lipid components on a Goldfisch extractor and making an appropriate dilution of the resulting egg oil in hexane for HPLC analysis.

TABLE 1

Equations for Calculating Cholesterol Concentration by HPLC

Range	Coefficient of correlation	Equation[a]
.1	.9969	$[(7.72 \times 10^{-6}) \times PA^{b}] + .11$
.2	.9998	$[(1.53 \times 10^{-5}) \times PA] + .09$
.5	.9957	$[(4.49 \times 10^{-5}) \times PA] + -2.54$
1.0	.9969	$[(10.9 \times 10^{-5}) \times PA] + -17.47$
Adjusted 1.0	.9947	$[)9.99 \times 10^{-5}) \times PA] + -5.13$

[a]Mg cholesterol = [(slope) × PA] + y intercept.
[b]PA, peak area.

RESULTS AND DISCUSSION

In HPLC, UV wavelengths can be used for cholesterol detection if there is no contribution of the solvent mobile phase to absorbance. The UV spectrum of cholesterol indicated that there is a broad peak below 220 nm with a wavelength maximum at 204 nm. Spectrophotometers have decreased sensitivity below 210 nm. Although many components absorb in the low UV range, 0.75% IPA in hexane provided a stable baseline for detecting cholesterol at 210 nm.

Figure 1 shows the chromatogram of purified cholesterol (A) and egg oil (B) from the HPLC system being used in this study. Peak 1 of Figure 1A was due to a difference in solvent of the standard and mobile phase. Based on previous use of this HPLC system (8) and on UV scan of the peak while in the detector, peak 1 of Figure 1B is the egg oil triglyceride. The retention time of cholesterol (peak 2) was around 5 min with 0.75% IPA/hexane. Tsai and Hudson (4) eluted cholesterol from a 30-cm Porasil column in less than 2.5 min with

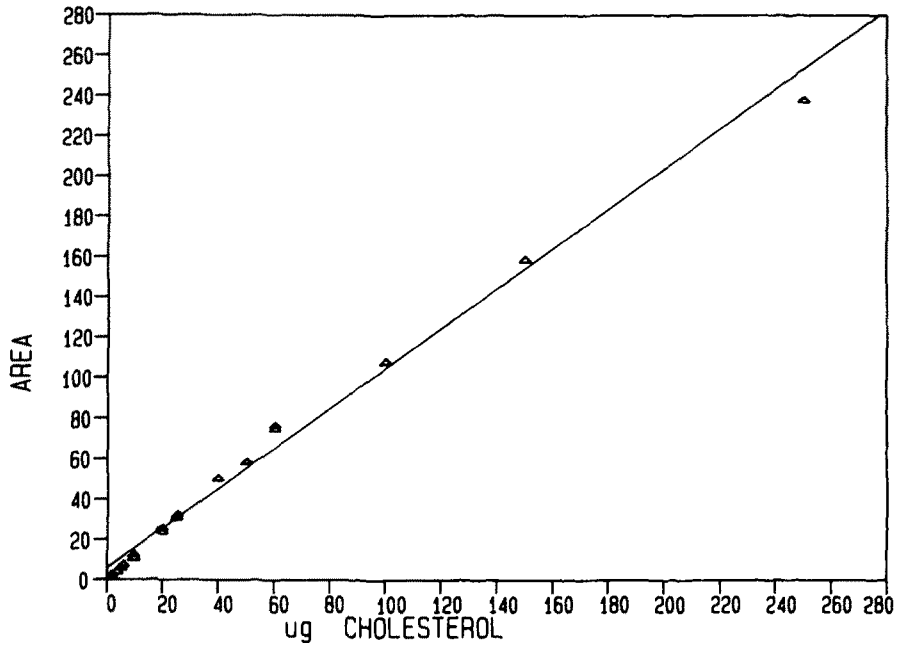

FIG. 3. HPLC calibration curve for cholesterol concentrations adjusted to 1.0 range.

3% IPA in hexane; their research showed that the more IPA in the hexane the shorter the retention time of the cholesterol. There was no difference in retention time of stigmasterol and cholesterol; thus, this technique should apply to tissues that have one predominant sterol.

In order to quantify the amount of cholesterol, aliquots of standard solutions ranging from 2 to 250 μg cholesterol were chromatographed. The plot of peak area (from the integrator) against concentration of cholesterol was found to be linear at each of the ranges of the detector, as shown in Figure 2. The equations for calculating concentration at each range are in Table 1. For the use of unknown concentrations, a standard equation would have to be used for each range. However, by adjusting the areas to range 1.0 (by dividing peak area by the reciprocal of the range) one linear plot (Fig. 3) and equation were obtained.

The accuracy of this technique was verified by comparing results of the HPLC analysis of cholesterol extracted with petroleum ether on a Goldfisch extractor with accepted values (9). Although there is a lot of variation in values for egg yolk cholesterol, Posati and Orr reported an average of 2,928 mg cholesterol per 100 g dried egg yolks in USDA Handbook 8-1 (9). In our laboratory we found 3,048 mg (standard deviation 117) cholesterol per 100 g spray-dried egg yolks in this lipid component extracted with petroleum ether. This HPLC technique can give a good estimate of the cholesterol content of egg yolks. Currently, research is being conducted on using the HPLC method to quantify cholesterol in other animal tissue.

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