Table II. Amino Acid Composition (mol %) of Proteins in *Brachychiton spp.*

amino acid	*B. discolor*	*B. diversifolius*	*B. acerifolius*
Asp	9.37	9.12	8.96
Thr	3.33	3.20	3.27
Ser	5.60	3.20	5.56
Glu	14.0	16.4	14.3
Pro	3.83	3.68	4.39
Gly	6.86	6.41	6.26
Ala	6.20	5.65	5.43
$^1/_2$Cys	0.87	1.05	1.05
Val	5.09	5.39	5.11
Met	1.24	1.44	1.07
Ile	3.52	3.64	3.59
Leu	6.29	6.38	5.85
Tyr	2.14	1.85	2.09
Phe	3.29	3.12	3.32
His	1.86	1.71	1.59
Lys	4.89	5.31	5.18
ammonia	11.6	12.3	11.2
Arg	9.95	7.41	11.9

crops such as soybeans, indicating their potential for future utilization as feed supplements. The values obtained for methionine and cystine are perhaps lower than the actual content since a small proportion of these acids could have been oxidized during acid hydrolysis and not shown in the chromatographic analysis. The edibility of the seed proteins and possible toxicity have yet to be ascertained.

ACKNOWLEDGMENT

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Registry No. Linoleic acid, 60-33-3; oleic acid, 112-80-1; malvalic acid, 503-05-9; sterculic acid, 738-87-4; dihydrosterculic acid, 5711-28-4.

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Overestimation of the Cholesterol Content of Eggs

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The current estimate of the cholesterol of eggs is based primarily on data obtained by colorimetric determinations, which are subject to interference from non-cholesterol substances. Studies were conducted to evaluate the cholesterol content of eggs by the use of high-performance liquid chromatography (HPLC) and to compare this method to a commonly used colorimetric procedure. The HPLC method resulted in a cholesterol content of 10.97 mg of cholesterol/g of wet yolk compared to 13.86 mg/g of wet yolk estimated by the colorimetric method on saponified yolk extracts. Recovery of added cholesterol to yolk samples was nearly quantitative. Separation of the cholesterol from the remaining unsaponifiable yolk fraction by HPLC revealed that 17.5% of the chromogens present in the colorimetric assay were in the non-cholesterol fraction. A reevaluation of the cholesterol content of eggs should be conducted using methods based upon prepurification of the cholesterol fraction from interfering chromogens prior to detection.

High blood plasma levels of cholesterol have long been associated wtih increased incidence of coronary heart disease in humans. Although the role of dietary consumption of cholesterol in heart disease is subject to considerable debate, consumer interest in lowering blood cholesterol levels has contributed to a steady decrease in the consumption of eggs and egg products. The estimated cholesterol content of 274 mg/egg set by the Consumer and Food Economics Institute of the U.S. Department of Agriculture (1976) is used as the current standard by the medical community to determine the recommended daily intake of cholesterol. This value was obtained by the compilation of data from various published and unpublished investigations prior to the last revision of this handbook in 1976, before more precise methods of cholesterol determination had become available.

Colorimetric determinations have been commonly used in the past to determine the cholesterol content of eggs although the absence of interfering compounds, which may increase the apparent cholesterol concentration, has not

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usually been verified. These procedures employ various extraction and saponification techniques resulting in an impure mixture of compounds to which the cholesterol determination is applied.

One of the more popular cholesterol determinations first introduced by Zlatkis et al. (1953) involves the reaction of cholesterol with a $FeCl_3$ reagent. Weiss et al. (1964) showed that this method considerably overestimates the cholesterol content of eggs in the presence of polyunsaturated fatty acids. Their data showed that the cholesterol content of eggs from hens fed 30% safflower oil or linseed oil was apparently increased 36 and 43%, respectively, when the direct Zlatkis et al. (1953) procedure was used. After saponification, the cholesterol contents of the eggs were only increased by 19 and 27%. Although these authors concluded that interference from carotenoids in the nonsaponifiable fraction was negligible, the isolated carotenoid fraction yielded a value of 8.5 mg of cholesterol/egg. Interferences from protein, steroids, turbidity (Brown, 1961), and vitamin A (Kinley and Krause, 1958) are known to decrease the accuracy of this procedure for estimating cholesterol.

Searcy and Bergquist (1960) proposed a similar method, substituting $FeSO_4$ for $FeCl_3$ in order to determine the absorbance at a wavelength less susceptible to interfering substances; however, this substitution failed to completely eliminate interference problems. The classical Liebermann–Burchard color reaction as reviewed by Zak and Ressler (1955) is well suited to automation (Technicon Methods, 1974) but is sensitive to light, temperature, and small amounts of moisture. Both the Zlatkis and Liebermann–Burchard procedures are further disadvantaged by the use of caustic reagents. Cholesterol may be precipitated with digitonin followed by determination with a colorimetric assay (Schoenheimer and Sperry, 1934; Sperry and Webb, 1950) although precipitation with substances other than cholesterol may occur (Bloor, 1943). Recently developed enzymatic procedures using cholesterol oxidase (EC 1.1.3.6) (Allain et al., 1974; Salé et al., 1984) are subject to a number of interfering steroids (Clark, 1980).

These procedures generally work well with purified cholesterol samples; however, when these techniques have been employed to determine the cholesterol content of egg extracts, there is an apparent increase in cholesterol content due to large amounts of other interfering substances present in eggs. In the following experiments, reversed-phase HPLC was used to separate and quantify the cholesterol from the remaining unsaponifiable material of yolk extract and to demonstrate the amount of interference of the separated fractions in the Zlatkis et al. (1953) procedure.

MATERIALS AND METHODS

Animals. Single-comb white leghorn hens, 60 weeks of age, were individually caged under artificial lights with 14 h light/day. Feed and water were provided ad libitum. A corn–soy diet formulated to meet NRC requirements (1984) without the addition of animal byproducts was fed 1 month prior to the initial egg sampling and throughout the study.

Sample Preparation. A sample of 30 eggs was weighed and boiled for 5 min. The yolks were removed, weighed, and thoroughly blended together. For studies using unboiled eggs, the yolks from a sample of 30 eggs were separated from the albumen, pooled, and thoroughly blended as before.

Yolk samples were extracted by the chloroform–methanol (2:1, v/v) method of Folch et al. (1956) as adapted by Washburn and Nix (1974). Saponification of the cholesterol esters was achieved by the method of Abell et al. (1952) with minor modifications. A 0.25-mL sample of the Folch extract was vortexed with 2.5 mL of freshly prepared 2% (w/v) potassium hydroxide in absolute ethanol. Samples were incubated at 37 °C for 1.5 h. The samples were extracted with 5.0 mL of petroleum ether followed by the addition of 2.5 mL of deionized water. The extract was then quickly placed in a tightly capped tube to prevent evaporation of the ether and thus concentration of the sample.

Table I. Recovery of Cholesterol from Extracted and Saponified Samples As Determined by HPLC

sample	n	mg added	rec, %
Folch extraction	5	2.5	94.8 ± 4.9[a]
	5	5.0	95.1 ± 2.8
saponification	5	0.125	96.2 ± 5.3
	5	0.250	96.3 ± 3.9

[a] Values are percent ± SD.

Cholesterol Analysis. A modification of the method of Duncan et al. (1979) was employed to determine the cholesterol content using HPLC. The HPLC apparatus consisted of a Model 7125 sample injector (Rheodyne, Inc., Cotati, CA) fitted with a 20-μL sample loop, a Model AA-100-S pump (Eldex Laboratories Inc., San Carlos, CA), a Spectroflow 773 absorbance detector (Kratos Analytical Instruments, Westwood, NJ), and an HP 3390A integrator (Hewlett-Packard, Avondsale, PA). The column was a 10-μm μBondapak C_{18} reversed-phase column (3.9 mm × 30 cm) (Waters Associates, Milford, MA) preceded by a C_{18} guard column (5 cm). The mobile phase buffer was 2-propanol–acetonitrile (1:1, v/v) pumped at 1.2 mL/min at 600–800 psi. All buffers and solvents were of HPLC grade and were filtered (0.45 μm) and degassed prior to use. The column eluate was monitored at 200 nm with 0.01 AUFS. Cholesterol obtained from the National Bureau of Standards (NBS) (Office of Standard Reference Materials, Washington, DC) was used as a standard. Aliquots of yolk extracts (0.5 mL) were dried under a stream of N_2 at room temperature, resuspended in 0.5 mL 100% 2-propanol, and filtered (0.45 μm) prior to injection into the HPLC. Identification and quantification of the eluted cholesterol standards and samples were done by comparing retention times and integration of the area under the peak. In the percent recovery experiments, NBS cholesterol standard was added to the yolk sample prior to Folch extraction and to the Folch extract prior to saponification. The percent recovery was determined by subtracting the average peak area of cholesterol present in unspiked samples from that in spiked samples.

The Zlatkis et al. (1953) procedure as modified by Weiss et al. (1964) and the unmodified Zlatkis procedure were performed on duplicate samples of the saponified extracts for comparison with the HPLC analysis. To demonstrate the presence of interfering substances in the yolk extracts, HPLC fractions of saponified yolk extracts were collected, pooled, and used in the Zlatkis procedure. Absorption spectra were obtained by the use of a scanning spectrophotometer (Spectronic 2000; Baush and Lomb, Rochester, NY). Data were analyzed by analysis of variance, with significant differences between means determined by Duncan's multiple-range test (SAS Institute, 1985).

RESULTS

High-performance liquid chromatography of up to 45.0 μg of NBS cholesterol standard resulted in a single peak with a retention time of 5.5 min. The standard curve was linear over 0.55–45 μg of cholesterol with $R^2 = 0.99$. A representative HPLC chromatogram of a boiled yolk extract shows complete base-line resolution of the cholesterol peak (Figure 1A). Samples with 1.0–20.5 μg of cholesterol resulted in similar chromatograms. The chromatogram from a sample of unboiled yolk extract revealed a similar profile (Figure 1B), indicating that boiling has no effect on the separation of the cholesterol from the remaining unsaponifiable extract.

Recovery of added cholesterol from the Folch extraction and saponification steps was nearly quantitative (Table I). The total recovery was greater than 94% when the cholesterol was added prior to the Folch extraction and greater than 96% when added prior to saponification. When cholesterol was added to the Folch and saponifica-

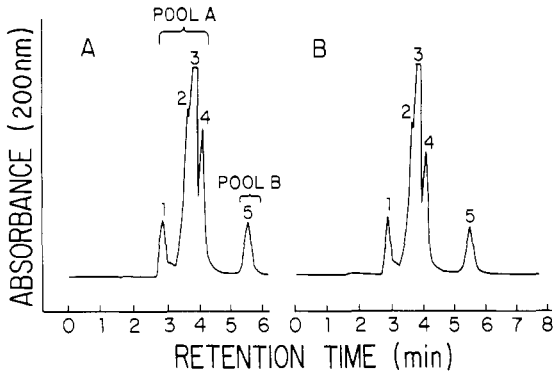

Figure 1. Representative HPLC chromatograms of (A) boiled yolk extract, 1.235 μg of cholesterol, and (B) unboiled yolk extract, 1.052 μg of cholesterol. There were no significant differences between the cholesterol content of unboiled egg yolks and that of boiled egg yolks. Peak identification: (1) 2-propanol; (2–4) unknown; (5) cholesterol.

Table II. Cholesterol Content of Pooled, Boiled Egg Yolks As Determined by Various Methods[a]

procedure	n	cholesterol[b] mg/g wet yolk	cholesterol[b] mg/egg[c]
Zlatkis, unsaponified	20	16.01[a] ± 0.71	289.8[a] ± 12.8
Zlatkis, saponified	20	13.86[b] ● 0.79	250.9[b] ± 14.2
HPLC	20	10.97[c] ± 0.32	198.6[c] ± 5.8

[a] Values within a column with different letters are significantly different ($P < 0.001$). [b] Values are means ± SD. [c] Based on 18.1-g average yolk weight.

Table III. Cholesterol Content of Nonchromatographed Control Samples and HPLC-Resolved Peaks As Determined by the Zlatkis Colorimetric Procedure

sample	n	cholesterol,[a] μg	%
nonchromatographed	20	29.3	100.0
chromatographed			
resolved pool A	20	5.2	17.5 ± 2.2[b]
resolved pool B	20	24.6	83.6 ± 2.6

[a] Values within a column represent the mean of a range of samples containing from 12.5 to 45.5 μg of cholesterol. [b] Values are percent ± SD.

tion steps in the absence of yolk sample, the percent cholesterol recovered was similar.

The cholesterol content as determined by various methods is shown in Table II. Multiple samples ($n = 20$) of boiled yolks yielded a cholesterol content of 10.97 mg of cholesterol/g of wet yolk with the HPLC procedure. When the colorimetric assay was run on unsaponified samples, the apparent cholesterol content was 46% higher than when estimated by the HPLC method. Saponified samples were 26% higher in cholesterol when estimated colorimetrically as compared to the HPLC estimate.

To determine the amount of interfering substances in the colorimetric assay, the cholesterol content of an HPLC-resolved sample was determined by the Zlatkis et al. (1953) procedure (Table III). The results indicate that pool A (peaks 1–4, Figure 1A) represents 17.5% of the total cholesterol content as determined by the Zaltkis et al. (1953) method.

The absorption spectra of the resolved sample (Figure 2) indicated that pool A contains substances that also absorb in the cholesterol range. The absorption spectra of an unresolved sample at the same concentration as the chromatographed sample show that the apparent cholesterol content is overestimated when the colorimetric procedure is run on unpurified egg extract.

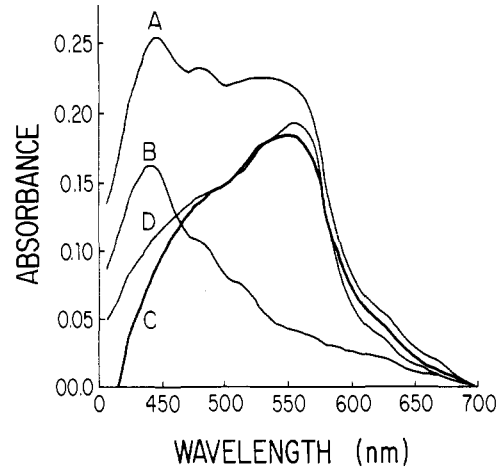

Figure 2. Absorption spectra of HPLC-resolved peaks as determined by the Zlatkis procedure: (A) unresolved sample; (B) pool of peaks 1–4, Figure 1A; (C) peak 5, Figure 1A; (D) standard cholesterol, 20.0 μg.

DISCUSSION

The accuracy of colorimetric methods for the determination of cholesterol in sera and eggs has been questioned for some time (Brown, 1961; Sweeney and Weihrauch, 1976). Due to the simplicity of these determinations, and owing to the fact that few alternatives were available, a large percentage of the data on the cholesterol content of eggs is based upon these determinations.

The data presented here show that purification of the cholesterol from egg yolk prior to quantification is necessary to obtain an accurate determination when compared to a common colorimetric procedure. Interfering chromogens are resolved from the cholesterol before detection and thus result in a more accurate determination. The higher value obtained by the colorimetric method on the unsaponified yolk extract has been suggested to be due to interference from polyunsaturated fatty acids (Weiss et al., 1964).

It has not been determined how many of the interfering chromogens may be procursors to cholesterol or oxidation and reduction artifacts created in the extraction and saponification procedures. Naber and Biggert (1985) found that saponification of the cholesterol esters results in the formation of oxidation products. This may decrease the apparent cholesterol content; however, their saponification procedure was quite harsh and the resultant increase of oxidation products small.

Recovery of free cholesterol added to yolk samples indicates that only 3.7–5.7% is lost during extraction and saponification. Similar recoveries of spiked cholesterol have been shown with egg noodles (Hurst et al., 1985) and milk (Hurst et al., 1983) with HPLC as the detection technique. The HPLC method could possibly underestimate the total cholesterol content if incomplete hydrolysis of the cholesterol esters occurs during saponification. It has been shown, however, that saponification to free cholesterol is complete under these conditions (Weiss et al., 1964).

The cholesterol content of eggs used in this study was found to be 27.5% lower in total cholesterol when determined by HPLC than that reported by the Consumer and Food Economics Institute (1976). This difference is largely due to the method of detection, but changes in husbandry, genetics, and diet formulation may also play some role in the lower value.

Dieticians generally use the value set by the Consumer and Food Economics Institute (1976) as a basis for de-

termining cholesterol intake to set the daily limit of 300 mg of cholesterol/day recommended by the American Heart Association (1986). Food composition tables have also been used to estimate the cholesterol content of eggs in studies to assess the effect of egg consumption on plasma lipid responses (Flynn et al., 1979; Applebaum-Bowden et al., 1979; Oh and Miller, 1985). Equations relating the dietary consumption of cholesterol to serum cholesterol levels have included data in which the cholesterol content of the eggs was calculated by the use of food composition tables (Hegsted, 1986; Keys, 1984). Therefore, overestimation of the cholesterol content of eggs may lead to errors in determining the effect of egg consumption. A reevaluation of the egg cholesterol content using more specific determinations should be undertaken. As demonstrated here, this value should be much lower, resulting in a more accurate assessment of the true cholesterol intake associated with egg consumption.

Registry No. Cholesterol, 57-88-5.

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