# Evaluation of Solid Phase Extraction and Gas Chromatography for Determination of Cholesterol Oxidation Products in Spray-Dried Whole Egg

Shu-Mei Lai,[†] J. Ian Gray,[*,†] and Matthew E. Zabik[‡]

Department of Food Science and Human Nutrition and Department of Entomology/Pesticide Research Center, Michigan State University, East Lansing, Michigan 48824

A method using solid phase extraction (SPE) and capillary gas chromatography (GC) was developed for the rapid quantification of cholesterol oxidation products (COPS) in egg powders. Total lipid extracts were fractionated on disposable silica SPE tubes to isolate COPS from triacylglycerols, phospholipids, and cholesterol. The COPS (α- and β-epoxides, 7α- and 7β-hydroxycholesterols, 7-ketocholesterol) were resolved as their trimethylsilyl ether derivatives on a nonpolar capillary column. Combined GC–mass spectrometry was used to confirm the identities of the trimethylsilyl ethers of COPS. Homogeneous and consistently high recoveries of COPS standards as well as 6-ketocholestanol (internal standard), approximately 86%, were achieved with this analytical technique.

**Keywords:** *Cholesterol oxidation products; quantitation; egg powder*

## INTRODUCTION

Numerous cholesterol oxidation products (COPS) possess biological activity, with some involved in atherogenesis, carcinogenesis, and cholesterol biosynthesis (Maerker, 1987; Kumar and Singhal, 1991). The adverse biological activities attributed to COPS have caused concern about their occurrence in foods (Finocchiaro and Richardson, 1983; Sander et al., 1989).

Over the past decade, various methods have been developed for the identification and quantification of COPS in food products exposed to heat and air during processing and/or storage. These include packed column gas chromatography (GC) (Morgan and Armstrong, 1987), capillary column GC (Park and Addis, 1985a; Missler et al., 1985; Nourooz-Zadeh, 1990; Morgan and Armstrong, 1992), and high-performance liquid chromatography (HPLC) (Tsai and Hudson, 1981; Park and Addis, 1985b; Sugino et al., 1986). However, there is no standard method that isolates and quantifies all major COPS in foods accurately and rapidly. This is partly due to the difficulties associated with the isolation of small amounts of COPS in foods that contain large amounts of interfering cholesterol, triacylglycerols, phospholipids, and other lipids (Missler et al., 1985; Park and Addis, 1985b; Nourooz-Zadeh, 1990).

Saponification has been commonly used as an enrichment step in the quantification of COPS in the presence of other lipids (Finocchiaro et al., 1984; Higley et al., 1986). However, hot alkaline treatment of cholesterol and COPS is a significant source of error in the quantification of COPS (Addis, 1986). Tsai et al. (1980) reported a loss of approximately 75% of the α-epoxide after saponification due to the hydrolysis of the epoxide ring. Substantial loss of 7-ketocholesterol also occurs as a result of saponification because of its high sensitivity to alkali and subsequent degradation to 3,5-cholestadien-7-one and several other products (Maerker and Unruh, 1986).

Recently, solid phase extraction (SPE) has been used intensively for COPS cleanup to avoid formation of artifacts and/or breakdown of COPS during saponification. Packed silicic acid columns and silica gel columns have been utilized to fractionate COPS from triacylglycerols and other lipids (Tsai and Hudson, 1984; Missler et al., 1985; Park and Addis, 1985b; Sugino et al., 1986). However, these columns cannot remove cholesterol substantially to avoid its interference with the quantification of some COPS, e.g., 7α-hydroxycholesterol (Morgan and Armstrong, 1987; Sander et al., 1989; Monahan et al., 1992). Furthermore, this column chromatographic procedure has cumbersome and tedious preparatory steps.

On the other hand, disposable columns or cartridges have become popular for sample cleanup because of their convenience and the improved reproducibility of the data obtained. Although they have been used effectively to enrich COPS from cholesterol and other lipids (Morgan and Armstrong, 1989, 1992; Nourooz-Zadeh, 1990), a broad range of recoveries (63.9–101.2%) of COPS has been reported with this method (Morgan and Armstrong, 1989). It has been suggested that some of the COPS may be selectively washed out to varying degrees with the cholesterol fraction during sample cleanup. However, no information about the capacity of the disposable columns or cartridges or the flow rate of solvents used for SPE is provided in these reports.

The objective of this study was to develop a method that can be used routinely for the quantification of all major COPS in egg powder. Some parameters that may affect the efficiency of SPE and the accuracy of quantification such as solvent flow rate and conditions for derivatization were also investigated.

## MATERIALS AND METHODS

**Reagents.** Cholesterol (cholest-5-en-3β-ol) and 6-ketocholestanol (6-oxo-5-cholestan-3β-ol) standards were purchased from Sigma Chemical Co. (St. Louis, MO). Cholesterol oxide standards, α- and β-epoxides (5α,6α-epoxycholestan-3β-ol and 5β,6β-epoxycholestan-3β-ol), 7α- and 7β-hydroxycholesterols (5-cholestene-3β,7α-diol and 5-cholestene-3β,7β-diol), 7-ketocholesterol (7-oxo-5-cholesten-3β-ol), 20α-hydroxycholesterol (5-

* Author to whom correspondence should be addressed [fax (517) 353-5406; e-mail 18215 JIG@msu.edu].

† Department of Food Science and Human Nutrition.

‡ Department of Entomology.

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cholestene-3$\beta$,20$\alpha$-diol), 25-hydroxycholesterol (5-cholestene-3$\beta$,25-diol), and cholestane-3$\beta$,5$\alpha$,6$\beta$-triol, were purchased from Steraloids Inc. (Wilton, NH). Bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA plus 1% TMCS) was obtained from Pierce Chemical Co. (Rockford, IL). Other reagents and solvents used in this study were of analytical grade and/or HPLC grade.

**Food Samples.** Whole liquid egg was spray-dried with a direct gas-heating spray-dryer (Kelly et al., 1989) at the National Dairy Products Research Centre (Fermoy, Cork, Ireland). The egg powders were vacuum packaged in polyethylene-laminated nylon pouches (Koch, Kansas City, MO) and immediately air freighted to Michigan. These pouches (90 $\mu$m thickness) have a water-vapor transmission rate of 0.041 mL m$^{-2}$ day$^{-1}$ mmHg$^{-1}$ and an oxygen transmission rate of 0.124 mL m$^{-2}$ day$^{-1}$ mmHg$^{-1}$ at 22.7 °C and 50% relative humidity. Once received (within 2 weeks of processing), the egg samples were repacked in low-density polyethylene bags (8 in. × 10 in., 75 $\mu$m thickness, Whirl-Pak, Fisher Scientific, Fair Lawn, NJ) without heat sealing and stored at ambient temperature (22 ± 2 °C) in the dark for 6 months. Sampling for analysis of the egg samples was performed randomly after the egg powder was thoroughly mixed in the bags with a spatula (approximately 100 g per bag).

**Extraction of Lipids.** Fifteen micrograms of the internal standard, 6-ketocholestanol, was added to 1.000 ± 0.005 g of egg powder prior to extraction. The sample was homogenized for 2 min with 30 mL of chloroform in a 100 mL beaker using an Ultra-Turrax type of homogenizer (Tekmar Co., Cincinnati, OH). The extract was then filtered through 5 g of anhydrous sodium sulfate and Whatman No. 4 filter paper to remove moisture and egg powder particles. Another 30 mL aliquot of chloroform was used to rinse the probe and to re-extract the residue. The combined extracts were evaporated to dryness with a vacuum rotary evaporator (Buchi Rotavapor, Postfach, Switzerland) and redissolved in 5 mL of hexane.

**Solid Phase Extraction.** The COPS in the lipid extract were isolated using a 3 mL Superclean LC-Si SPE tube (Supelco, Bellefonte, PA) filled with 300 mg of silica packing (40 $\mu$m particles, 60 Å pores). The SPE tube was wetted with 3 mL of hexane to activate the packing before the sample was added. After sample addition, the following solvent combinations were applied to the SPE tube when approximately 1 mm of the previous solvent remained above the top of the tube frit: 10 mL of hexane/ethyl ether (95:5 v/v), 25 mL of hexane/ethyl ether (90:10 v/v), and 15 mL of hexane/ethyl ether (80:20 v/v). Finally, the COPS were eluted out of the column with 5 mL of acetone. Chromatography was accelerated with a vacuum of 20 kPa using a vacuum manifold (Supelco) which was attached to a water-suction arrangement. The vacuum manifold also controlled the flow rate of the solvents (0.6 ± 0.1 mL/min). This system, through the use of independent screw-type valves, permitted the simultanepus extraction of up to 12 samples.

**Derivatization of COPS to TMS Ethers.** After the solvent was evaporated under a stream of nitrogen, the COPS were redissolved in 100 $\mu$L of BSTFA plus 1% TMCS in a half-dram glass vial, the vial was capped, and the contents were mixed with a vortex mixer for 30 s. This mixture was placed in the dark at room temperature for 50 min to form the trimethylsilyl (TMS) ether derivatives of the COPS. Subsequently, the TMS reagent was removed under nitrogen to dryness, and the residue was dissolved in 100 $\mu$L of hexane.

**GC Analysis.** TMS ethers of COPS were analyzed using a Hewlett-Packard (HP) 5890A GC (Avondale, PA) equipped with a flame ionization detector. A 15 m × 0.25 mm i.d. DB-1 (0.1 $\mu$m film thickness) capillary column (J&W Scientific Inc., Folsom, CA) operated with a helium carrier gas (column flow rate of 1 mL/min) was used for the separation of the TMS ethers. The oven temperature was programmed from 170 to 220 °C at a rate of 10 °C/min and then increased to 236 °C at a rate of 0.4 °C/min. After the peaks of interest (COPS) were eluted, the oven temperature was increased at a rate of 10 °C/min to a final temperature of 300 °C and held for 25 min or until all lipid residues were eluted out of the column. The temperatures of the injection port and detector were held at 275 and 320 °C, respectively. Two microliters of the TMS derivatives of the COPS was injected onto the column with a split ratio of 11:1. Peak areas were integrated with a HP 3392A integrator and converted to quantity of COPS using the internal standard method.

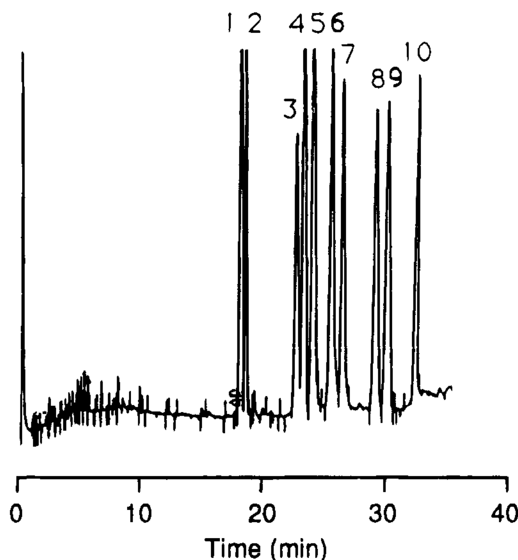


**Figure 1.** Gas chromatogram of TMS ethers of cholesterol oxide standards; peak numbers correspond to those listed in Table 1.

**GC-Mass Spectrometry (GC-MS).** A HP 5890A GC equipped with a 5970A mass selective detector was employed. GC-MS analyses were performed using the same capillary column and temperature programming as for the quantification of COPS. The operation conditions were as follows: ion source temperature, 200 °C; electron voltage, 70 eV; and electron multiplier, 2000 V. The mass spectra of the TMS ether derivatives of COPS, scanned within the mass range $m/e$ 50-650, were recorded.

**Recovery of COPS.** Recovery studies on COPS through the SPE procedures were performed with various concentrations of the COPS standards. Each recovery experiment was repeated three times, and the extracts were analyzed in duplicate.

## RESULTS AND DISCUSSION

**Isolation and Identification of COPS.** Incomplete separation by capillary GC of some COPS or overlapping pairs, such as cholesterol and 7$\alpha$-hydroxycholesterol (Park and Addis, 1986; Sander et al., 1989) and the isomeric forms of 5,6-epoxides (Fisher et al., 1985), has been a major difficulty in the quantification of COPS. In this study, the complete separation of all relevant COPS was achieved by nonpolar capillary column (DB-1) gas chromatography after extensive evaluation of the gas chromatographic parameters. Separation of cholesterol, 7$\alpha$- and 7$\beta$-hydroxycholesterols, $\alpha$-epoxide, $\beta$-epoxide, 7-ketocholesterol, 20$\alpha$-hydroxycholesterol, 25-hydroxycholesterol, and cholestane-3$\beta$,5$\alpha$,6$\beta$-triol standards chromatographed as their TMS ethers is shown in Figure 1. Retention times and retention times relative to that of the internal standard, 6-ketocholestanol, are presented in Table 1.

Although 5$\alpha$-cholestane has been frequently used as the internal standard for the quantification of COPS (Park and Addis, 1986; Sander et al., 1989), under our conditions it was eluted out of the column after 10 min and interfered with other BSTFA byproducts. For this reason, 6-ketocholestanol was selected as the internal standard because it was found to elute between 7$\beta$-hydroxycholesterol and 7-ketocholesterol and did not

**Table 1. Retention Times and Relative Retention Times[a] for Cholesterol and Cholesterol Oxidation Products**

| peak no. | compd | retention time (min) | relative retention time (min) |
|---|---|---|---|
| 1 | cholesterol | 18.4 | 0.59 |
| 2 | 7α-hydroxycholesterol (7α-OH) | 19.3 | 0.62 |
| 3 | 5β,6β-epoxycholesterol-3β-ol (β-epoxide) | 23.7 | 0.76 |
| 4 | 5α,6α-epoxycholesterol-3β-ol (α-epoxide) | 24.2 | 0.78 |
| 5 | 7β-hydroxycholesterol (7β-OH) | 24.9 | 0.80 |
| 6 | 20α-hydroxycholesterol (20α-OH) | 27.2 | 0.87 |
| 7 | 25-hydroxycholesterol (25-OH) | 28.0 | 0.90 |
| 8 | 6-ketocholestanol (6-keto) | 31.1 | 1.00 |
| 9 | 7-ketocholesterol (7-keto) | 32.0 | 1.03 |
| 10 | cholestane-3β,5α,6β-triol (triol) | 33.5 | 1.08 |

[a] Retention time relative to the internal standard (IS), 6-ketocholestanol (6-keto).

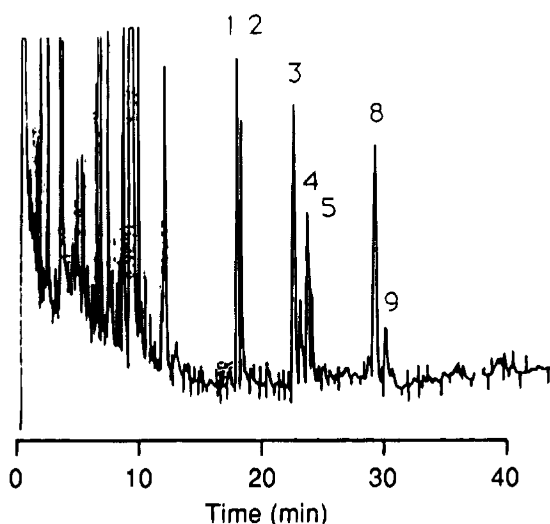


**Figure 2.** Gas chromatogram of TMS ethers of cholesterol oxidation products from egg powder; peak numbers correspond to those listed in Table 1.

interfere in the quantification of other oxides during GC and GC–MS analyses. In addition, it is structurally similar to 7-ketocholesterol, yet it has not been reported as a product of cholesterol oxidation in foods (Smith, 1981).

The identification of COPS in egg samples was based on the relative retention times and mass spectra of sample TMS ethers compared to those of the relevant COP standards. The gas chromatogram of a spray-dried egg sample (Figure 2) showed separations of COPS as well as cholesterol. This indicated that the cleanup procedure removed sufficient amounts of cholesterol to prevent interference with the quantification of COPS.

The mass spectra of TMS ethers of 7α- and 7β-hydroxycholesterols, α- and β-epoxides, and 7-ketocholesterol, 20α-hydroxycholesterol, 25-hydroxycholesterol, and cholestane-3β,5α,6β-triol standards were in agreement with those published in the literature (Brooks et al., 1973; Park and Addis, 1985a, 1986, 1987). On the basis of the molecular ion peaks of TMS sterols (Table 2), COPS with one or two hydroxyl groups were identified as the corresponding mono- and bis-TMS ethers, respectively. The presence of characteristic peaks corresponding to M − 90 and M − 180 in TMS sterols supported the identity of sterols as either the mono- or bis-TMS ethers, in accordance with the number of the hydroxyl groups.

**Table 2. Mass Spectrometric Data for TMS Ethers of Cholesterol and Cholesterol Oxidation Products**

| TMS ether | characteristic ion | | | | | | |
|---|---|---|---|---|---|---|---|
| | B[a] | M[b] | M − 15 | M − 90 | M − 105 | M − 180 | M − 195 |
| cholesterol | 129 | 458 (42)[c] | 443 (12) | 368 (77) | 353 (36) | | |
| 7α-OH | 456 | 546 (2) | 531 (1) | 456 (100) | 441 (2) | 366 (1) | 351 (1) |
| 7β-OH | 456 | 546 (1) | 531 (1) | 456 (100) | 441 (1) | 366 (2) | 351 (1) |
| α-epoxide | 73 | 474 (15) | 459 (8) | 384 (16) | 369 (6) | | |
| β-epoxide | 73 | 474 | 459 (35) | 384 (8) | 369 (35) | (12) | |
| 7-keto | 472 | 472 (100) | 457 (17) | 382 (20) | 367 (34) | | |
| 20α-OH | 69 | 546 (3) | 531 (1) | 456 (7) | 441 (2) | 366 (3) | 351 (5) |
| 25-OH (mono-) | 129 | 474 (25) | 459 (8) | 384 (50) | 369 (13) | | |
| 25-OH (bis-) | 131 | 546 (2) | 531 (1) | 456 (6) | 441 (2) | 366 (2) | 351 (2) |
| triol (bis-) | 75 | 564 (2) | | 474 (12) | 459 (5) | 384 (9) | 369 (7) |
| triol (tris-) | 75 | 636 (2) | | 546 (6) | 531 (2) | 456 (18) | 441 (4) |

[a] Base peak. [b] Molecular ion. [c] Percentage of base peak.

Comparison of the relative GC retention times and fragmentation patterns of mass spectra of COPS peaks from the egg samples with those of COPS standards led to the identification of five COPS. They were α- and β-epoxides, 7α- and 7β-hydroxycholesterols, and 7-ketocholesterol. These compounds have been reported in egg samples previously. For example, Nourooz-Zadeh and Appelqvist (1987) reported that after 8 years of storage, a dehydrated egg yolk sample contained these COPS plus 20α-hydroxycholesterol, 25-hydroxycholesterol, and cholestane-3β,5α,6β-triol. Missler et al. (1985) detected 25-hydroxycholesterol and cholestane-3β,5α,6β-triol in dried egg mixes that had been stored in cans for 5 years. However, 20α-hydroxycholesterol and 25-hydroxycholesterol, the products of side chain oxidation of cholesterol, as well as cholestane-3β,5α,6β-triol, the hydration products of cholesterol epoxides, were not detected in the spray-dried whole egg powders that were stored at room temperature for 6 months in this study.

**Derivatization and Response Factors of COPS.** The thermal decomposition of cholesterol diols such as 7α- and 7β-hydroxycholesterols and 25-hydroxycholesterol has been observed during GC analysis (Park and Addis, 1985a). The instability of COPS during GC analysis can be overcome by converting the COPS to their TMS derivatives (Park and Addis, 1985a). To set the optimum silylating conditions for COPS, BSTFA plus 1% TMCS was used under different time–temperature conditions (room temperature and 60 °C for 0.5, 1, 2, 6, 12, and 24 h) to convert COPS to their TMS ethers.

The maximum peaks areas for 7α- and 7β-hydroxycholesterols, α- and β-epoxides, 6-ketocholestanol, and 7-ketocholesterol were reached when they were derivatized with BSTFA plus 1% TMCS alone for 1 h. However, GC–MS analysis of the TMS ethers of COPS standards revealed incomplete derivatization of some COPS with this derivatization procedure. The 25-hydroxycholesterol was converted to the mono-TMS derivatives, with the TMS ether formed at the 3-position. The cholestane-3β,5α,6β-triol was found to form the bis-TMS ether with the hydroxyl groups at the 3- and 6-positions being derivatized.

**Table 3. Response Factor[a] and Percent Recovery of Cholesterol Oxidation Products (COPS)**

| | response factor | | % recovery | |
|---|---|---|---|---|
| COPS | mean[b] | CV (%) | mean[c] | CV (%) |
| 7α-OH | 1.9356 | 5.7 | 86.7 | 2.9 |
| β-epoxide | 1.6582 | 5.1 | 84.9 | 3.5 |
| α-epoxide | 1.0261 | 3.0 | 84.3 | 2.5 |
| 7β-OH | 1.0471 | 2.1 | 85.0 | 2.2 |
| 20α-OH | 1.0842 | 7.2 | 86.3 | 3.6 |
| 25-OH | 1.4800 | 8.0 | 86.8 | 5.2 |
| 6-keto | 1.0000 | 0.0 | 86.8 | 3.5 |
| 7-keto | 1.0788 | 1.8 | 84.2 | 2.7 |
| triol | 1.5000 | 6.2 | 86.3 | 4.6 |

[a] Response factor = $(W_i/A_i)/(W_{is}/A_{is})$; $W_i$ and $W_{is}$ are weights of COPS and internal standard (6-keto), respectively; $A_i$ and $A_{is}$ are peak areas of COPS and IS. [b] Mean of six replicated experiments (i.e., derivatization and GC analysis). [c] Mean of three replicated experiments (i.e., solid phase extraction, derivatization, and GC analysis).

With increase of derivatization time and/or temperature, the peak areas of the mono-TMS derivative of 25-hydroxycholesterol and the bis-TMS ether of cholestane-3β,5α,6β-triol decreased, while the peaks corresponding to the bis-TMS ether of 25-hydroxycholesterol and the tris-TMS ether of cholestane-3β,5α,6β-triol appeared at retention times of 33 and 31.5 min, respectively. The presence of two TMS derivatives for 25-hydroxycholesterol or cholestane-3β,5α,6β-triol could be a source of error in quantification and thus is not desirable.

Complete derivatization of 25-hydroxycholesterol and cholestane-3β,5α,6β-triol to their bis- and tris-TMS ethers was achieved by silylating these COPS with BSTFA plus 1% TMCS at 60 °C for 1 h or at room temperature for 24 h. However, decreases in the peak areas of other COPS were observed when complete derivatization of 25-hydroxycholesterol and cholestane-3β,5α,6β-triol was performed. Missler et al. (1985) indicated that the harsher silylating conditions used to completely derivatize 25-hydroxycholesterol and cholestane-3β,5α,6β-triol resulted in cleavage of the epoxide bonds in cholesterol epoxide isomers and formation of enol-TMS derivatives from the COPS with ketone groups. Decomposition of COPS derivatives when using a stronger silylating agent such as Sylon BTZ [*N*-(trimethylsilyl)imidazole + *N*,*O*-bis[trimethylsilyl]acetamide + TMCS, 3:3:2 v/v/v] has also been reported by Nawar et al. (1991).

On the other hand, BSTFA with 1% TMCS as an acid catalyst not only gave maximum peak areas for COPS with free hydroxyl groups such as 7α- and 7β-hydroxycholesterols, α- and β-epoxides, 6-ketocholestanol and 7-ketocholesterol but also provided the best combination of good peak shape and separation during the GC analysis of COPS. Therefore, in this study, isolated COPS were derivatized using BSTFA with 1% TMCS at room temperature for 50 min and analyzed by capillary GC. The average response factors of COPS for this study are listed in Table 3. The response factors of COPS were the ratios of absolute response factor (weight divided by peak area) of each COP to that of the internal standard. Some investigators have assumed that the response factor for all COPS is 1 (Park and Addis, 1985a; Nourooz-Zadeh, 1990) when quantifying the amounts of COPS in foods. Investigators should be aware that the response factor for each COP may vary with the different derivatization conditions, type of GC column, and GC operating conditions. Therefore, the response factors of COPS should be calculated for each individual study instead of using the values cited in the literature.

**Evaluation of SPE Cleanup.** The average lipid content of whole egg powder is approximately 40%, triacylglycerol and phospholipids being the major components. The cholesterol content in egg lipids ranges from 2 to 6.2%, with approximately 90% being present in the free form (Nourooz-Zadeh, 1990). On the other hand, the concentration of each COP in egg powder only ranges from 0 to 100 μg/g (Tsai and Hudson, 1985). Therefore, it is necessary to isolate COPS from other major lipids and increase their relative concentration prior to GC analysis.

In this study, SPE was used to separate COPS from other lipids and cholesterol according to their differences in polarity. Lipid components in order of increasing polarity are esterified cholesterol, triacylglycerols, free fatty acids, cholesterol, COPS, and phospholipids (Park and Addis, 1985b). Egg lipids extracted from egg powder (1 g) were dissolved in hexane, a nonpolar solvent, and then applied to a disposable SPE tube containing a highly polar silica packing. To ascertain the capacity of SPE tubes for retaining cholesterol and COPS, egg lipid extracts were applied to SPE tubes filled with various amounts of silica packing, 100, 300, and 500 mg. Results indicated that 300 mg of packing effectively held up to 500 mg of lipids without elution of any COP with hexane or solvent combinations of hexane and ethyl ether.

After the less polar lipids such as triacylglycerols and cholesterol were eluted out of the SPE tube using hexane/ethyl ether combinations of slightly increasing polarity, acetone was used to recover all COPS, while leaving most of the phospholipids on the column. A solvent flow rate of approximately 0.6 mL/min was maintained by a vacuum manifold throughout the chromatographic process.

Five milliliter fractions of solvent were collected to investigate the distribution of cholesterol and COPS during SPE. GC analyses showed that more than 99% of the cholesterol was eluted using the 90:10 and 80:20 hexane/ethyl ether solvents. The acetone fraction contained all recovered COPS and only small amounts of cholesterol (approximately 20 μg/g), which could be completely separated from 7α-hydroxycholesterol during GC analysis.

Because cholesterol and COPS have similar polarities, it is difficult to remove enough cholesterol to prevent its interference in the quantification of COPS without losing any of the latter compounds at the same time. Missler et al. (1985) used preparative HPLC to further remove excess cholesterol from samples after an initial sample cleanup with silica gel column chromatography and prior to final GC analysis. They reported that with this method 20% of the 25-hydroxycholesterol present was also eluted out with the cholesterol fraction. Nourooz-Zadeh (1990) used three enrichment steps to isolate COPS from egg samples. First, $NH_2$ cartridges were used to separate cholesterol and COPS from triacylglycrols and phospholipids. The eluate from the $NH_2$ cartridge containing cholesterol and COPs was applied to a Sep-Pak $C_{18}$ cartridge. After the second enrichment step, most of the COPS (>90%) and 40% of the cholesterol were recovered. Prior to GC analysis, preparative HPLC on a cyanopropyl column was used for the removal of the remaining cholesterol from COPS. In the present study, a single-step cleanup using a silica-packed SPE tube and a well-controlled solvent

flow rate not only removed cholesterol and other lipids sufficiently to avoid the interference with the quantification of COPS in egg powder but also gave reproducible and satisfactory recoveries (86% + 3%) for the internal standard (6-ketocholestanol).

To study the recovery of each COP in the SPE procedure, standard mixes of nine COPS at three different concentrations (10, 50, 100 $\mu g$ per COP) in hexane were applied to the SPE tubes containing 300 mg of silica packing, and the acetone fractions were collected, derivatized, and analyzed by capillary GC. No significant differences ($P < 0.05$) in percent recoveries of COPS at different concentrations were found. Therefore, data at all levels were combined and averaged (Table 3). The percent recoveries of COPS ranged from 84.2% to 86.8% with coefficients of variation (CV) from 2.2% to 5.2%.

The percent recoveries of COPS reported in the literature range from 23.6% (Higley et al., 1986) to nearly 100% (Park and Addis, 1985b), although only two or three COPS were quantified in each report. Morgan and Armstrong (1989) developed a wide-bore capillary GC method to quantify the five most common COPS in egg powder after COPS were cleaned up using a Sep-Pak silica cartridge. They reported average recoveries of 78.2% and 95.1% for α-epoxide and 7-ketocholesterol, respectively. In addition, large variations within each concentration of COPS as well as among different COPS were observed with this method.

Homogeneous ($P < 0.05$) and sufficiently high recoveries of COPS were achieved in this study with SPE tubes and solvent systems similar to those used by Morgan and Armstrong (1989). The solvent flow rate was controlled by a vacuum manifold with a vacuum of 20 kPa to provide a slow and constant flow rate (0.6 ± 0.1 mL/min). No information about the flow rate of solvents has been reported in the other studies in which SPE was used to isolate COPS from other lipids and/or cholesterol. Therefore, the large variations among the recoveries of COPS reported in the literature may be attributed to inconsistent solvent flow rates. In addition, this system, in which up to 12 samples can be cleaned up simultaneously in 1.5 h, is relatively faster than the conventional chromatographic cleanup procedure (Monahan et al., 1992), which takes approximately 3 h to prepare columns and extract four samples.

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