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Abstract

A study was conducted to develop a solvent system that will clean egg yolk samples and concentrate cholesterol oxides effectively before analysis. Cholesterol oxide standards or lipid samples (0.2 g) loaded onto a silicic column were washed with a portion of Solvent I (hexane/diethyl ether, 9:1, vol/vol) and then with Solvent II. Four different Solvent II preparations (Solvent IIa, hexane:ethyl acetate = 4:1; Solvent IIb, hexane:ethyl acetate = 1:1; Solvent IIc, hexane:ethyl acetate:diethyl ether = 2:1:1; Solvent IId, hexane:ethyl acetate:diethyl ether = 4:1:2, vol/vol/vol) were prepared and the purification efficiencies of Solvent II solutions for neutral lipids, cholesterol, and phospholipids in the column were compared. Yield study using cholesterol oxide standards showed that one or more of the cholesterol oxide standards were eluted by the Solvent IIb and Solvent IIc, but Solvent IIa and Solvent IId did not elute any of the cholesterol oxides during washing. Egg samples prepared with Solvent IIa showed greater amount of cholesterol oxides than those prepared with Solvent IId, probably due to incomplete purifying of phospholipids and interference. However, the amounts of cholesterol oxides in cooked meat prepared with the two purification solvents were not different. Because egg yolk contains very large amounts of phospholipids and cholesterol compared with other foods, at least twice as much Solvent IIa as Solvent IId was required to properly clean egg yolk samples. It was concluded that purification solvents should be selected by sample types, and Solvent IId (hexane:ethyl acetate:diethyl ether = 4:1: 2) was superior to Solvent IIa (hexane:ethyl acetate = 4: 1) for egg yolk samples.

Keywords

cholesterol oxides, purification solvents, egg yolk, cholesterol, phospholipids

Disciplines

Agriculture | Animal Sciences | Poultry or Avian Science

Comments

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Analysis of Cholesterol Oxides in Egg Yolk and Turkey Meat[1]

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ABSTRACT A study was conducted to develop a solvent system that will clean egg yolk samples and concentrate cholesterol oxides effectively before analysis. Cholesterol oxide standards or lipid samples (0.2 g) loaded onto a silicic column were washed with a portion of Solvent I (hexane/diethyl ether, 9:1, vol/vol) and then with Solvent II. Four different Solvent II preparations (Solvent IIa, hexane:ethyl acetate = 4:1; Solvent IIb, hexane:ethyl acetate = 1:1; Solvent IIc, hexane:ethyl acetate:diethyl ether = 2:1:1; Solvent IId, hexane:ethyl acetate:diethyl ether = 4:1:2, vol/vol/vol) were prepared and the purification efficiencies of Solvent II solutions for neutral lipids, cholesterol, and phospholipids in the column were compared. Yield study using cholesterol oxide standards showed that one or more of the cholesterol oxide standards were eluted by the Solvent IIb and Solvent IIc, but Solvent IIa and Solvent IId did not elute any of the cholesterol oxides during washing. Egg samples prepared with Solvent IIa showed greater amount of cholesterol oxides than those prepared with Solvent IId, probably due to incomplete purifying of phospholipids and interference. However, the amounts of cholesterol oxides in cooked meat prepared with the two purification solvents were not different. Because egg yolk contains very large amounts of phospholipids and cholesterol compared with other foods, at least twice as much Solvent IIa as Solvent IId was required to properly clean egg yolk samples. It was concluded that purification solvents should be selected by sample types, and Solvent IId (hexane:ethyl acetate:diethyl ether = 4:1:2) was superior to Solvent IIa (hexane:ethyl acetate = 4:1) for egg yolk samples.

(*Key words*: cholesterol oxides, purification solvents, egg yolk, cholesterol, phospholipids)

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INTRODUCTION

Cholesterol is known to undergo autoxidation during processing and storage, and cholesterol oxides have been found in several foods including egg (Zunin *et al*., 1995), milk (Chan *et al*., 1993), meat (Pie *et al*., 1991), and marine products (Osada *et al*., 1993). Over the past two decades, various methods have been developed to quantify and identify cholesterol oxides in food products. Saponification (hot and cold) or saponification-thin layer chromatography (TLC) had been widely used by earlier workers as isolation and prefractionation steps of cholesterol oxides before HPLC or gas chromatography (GC) analysis. However, saponification and TLC are no longer being used because of low recovery rates, destruction of certain cholesterol oxides, and oxidation of cholesterol during the procedure (Tsai and Hodson, 1985; Maerker and Unruh, 1986; Csallany *et al*., 1989). Currently, extraction of lipids with chloroform or chloroform:methanol (2:1) solution followed by sample purification steps on a silicic column is the most widely used method for cholesterol oxides preparation (Park and Addis, 1985; Lai *et al*., 1995a; Li *et al*., 1996). Among the steps, purification of samples is the most critical for the successful analysis of cholesterol oxides in foods because it allows concentration of cholesterol oxides and eliminates interference by neutral lipids, cholesterol, and phospholipids during GC analysis.

One or several solvent mixtures have been used to remove neutral lipids, phospholipids, and cholesterol during the purification step before the collection of cholesterol oxides (Tsai and Hodson, 1985; Bovenkamp *et al*., 1988; Kim and Nawar, 1991; Zubillaga and Maerker 1991; Schmarr *et al*. 1996). Two-step washing of silicic column with hexane/diethyl ether (9:1, vol/vol) and hexane/diethyl ether (8:2, vol/vol) is among the most frequently used methods (Park and Addis, 1985; Li *et al*., 1996) and has proven to be effective in removing neutral lipids and cholesterol from meat lipids (Li *et al*.,

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Abbreviation Key: BHT = butylated hydroxytoluene; BSTFA = *bis*-[trimethylsilyl]trifluoroacetamide; FID = flame ionization detector; GC = gas chromatography; TLC = thin layer chromatography; TMCS = trimethylchlorosilane.

1996). However, preliminary results in our laboratory indicated that purifying egg yolk samples with the hexane / diethyl ether system was very difficult because of high cholesterol and phospholipid content in egg yolk. The objective of this study was to develop a solvent system that will remove cholesterol and phospholipids effectively without eluting any cholesterol oxides from egg yolk lipids.

MATERIALS AND METHODS

Chemicals and Solvent Mixtures

Cholesterol and cholesterol oxide standards, including cholesterol (5-cholesten-3β-ol), 5α-cholestane, 19-hydroxycholesterol (5-cholestene-3β, 19-diol), 7β-hydroxycholesterol (5-cholestene-3β, 7β-diol), 20α-hydroxycholesterol (5-cholestene-3β, 20α-diol), α-epoxide (5α, 6α-epoxycholestan-3β-ol), β-epoxide (5β, 6β-epoxycholestan-3β-ol), cholestanetriol (3β, 5α, 6β-tritrihydroxycholestane), 25-hydroxycholesterol (5-cholestene-3β-25-diol), 22-ketocholesterol (5-cholesten-3β-ol-22-one), 6-ketocholestanol (5α-cholestan-3β-ol-6-one), 7-ketocholesterol (5-cholesten-3β-ol-7-one), and butylated hydroxytoluene (BHT) were purchased from Sigma.[3] *bis*-[trimethylsilyl]trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) was obtained from Supelco.[4] Celite-545 and calcium phosphate ($CaHPO_4 \cdot 2H_2O$) were purchased from Fisher,[5] and silicic acid (100 to 200 mesh) was from Aldrich.[6] Hexane, ethyl acetate, ethyl ether, acetone, and methanol were HPLC grade.

Solvent I (hexane:ethyl acetate = 9:1, vol / vol), Solvent IIa (hexane:ethyl acetate = 4:1, vol / vol), and Solvent III (acetone:ethyl acetate:methanol = 10:10:1, vol / vol / vol) were prepared and used in the reference method (Li *et al*., 1996). Solvent IIb (hexane:ethyl acetate = 1:1, vol / vol), Solvent IIc (hexane:ethyl acetate:ethyl ether = 2:1:1, vol / vol / vol), and Solvent IId (hexane:ethyl acetate: ethyl ether = 4:1:2, vol / vol / vol) were prepared for the modified purification methods.

Sample Preparation

Egg yolk powders from four different lots (replications) were obtained from a local egg processor and packaged in eight oxygen-permeable plastic bags (two bags per each lot). Four bags of egg yolk powders (each from different lot) were stored at room temperature (22 C) and the other four at refrigerated temperature (4 C) for 8 m. Turkey thigh muscles were separated from four turkeys, and skin and visible fat were removed. The thigh meat from each turkey was ground separately through a 3-mm plate and eight meat patties (approximately 100 g each) were prepared from each turkey. The meat from each of the four turkeys represented four experimental replications. Patties were cooked in a 300 C electric oven to an internal temperature of 78 C, packaged in oxygen-permeable bags, and then analyzed after 0 and 7 d of storage at 4 C.

Lipid Extraction

Lipids were extracted from egg yolk powder and turkey thigh meat according to the method of Folch *et al*. (1957). Each sample (1 g egg yolk powder or 5 g turkey meat), BHT (50 μL, 7.2%), and 30 mL Folch solution ($CHCl_3$:CH_3OH = 2:1), were added to a 50 mL test tube and homogenized using a Polytron[7] for 20 s (speed set at 7 to 8). The homogenate was filtered through a Whatman No. 1 filter paper[8] into a 100-mL graduated cylinder, and the filter paper was rinsed twice with 10 mL Folch 1 solution. After adding 8 mL of 0.88% NaCl solution to each cylinder, the cylinder was capped with a glass stopper and the content mixed. The inside of cylinder was washed twice with 5 mL of Folch 2 solution (3:47:48 / $CHCl_3$:CH_3OH:H_2O). After phase separation, the lipid layer volume was recorded, and top layer (methanol and water) of the solution was completely and carefully siphoned off so as not to contaminate $CHCl_3$ layer. The organic layer was put in a glass scintillation vial and dried in a block heater[3] (1 h at 50 C). The dried lipid was dissolved with an aliquot of hexane to make 0.1 g fat / mL hexane and used for the next step.

Sample Purification and Preparation of Cholesterol Oxidation Products

Column Preparation. Silicic acid (100 mesh), celite-545, and $CaHPO_4 \cdot 2H_2O$ (10:9:1, wt / wt / wt) mixture in chloroform was prepared and packed into a glass column (22 mm $\times$ 30 cm with a sintered glass frit at the bottom) to a height of 10 cm. The column was washed with 10 mL of hexane:ethyl acetate (9:1 vol / vol, Solvent I) before loading a sample.

Sample Preparation. Lipid sample dissolved in hexane (0.2 g) was loaded onto the silicic acid column. Neutral lipids, cholesterol, and phospholipids were eluted by passing 40 mL of Solvent I (hexane:ethyl acetate = 9:1, vol / vol) and 40 or 80 mL Solvent II (80 mL for Solvent IIa, and 40 mL for Solvent IIb, Solvent IIc, and Solvent IId) through the column. Then cholesterol oxides were eluted with 40 mL of Solvent III (acetone:ethyl acetate:methanol = 10:10:1, 1 mL / min flow rate) and dried under nitrogen. The dried cholesterol oxides were added with 200 μL pyridine and 100 μL BSTFA+1% TMCS and derivatized by heating in a dry bath (80 C) for 1 h. For modified purification methods, three different Solvent II were tested for their purification efficiency for neutral lipids, cholesterol, and phospholipids in the column.

[3]Sigma Chemical Co., St. Louis, MO 63178-9916.
[4]Supelco, Bellefonte, PA 16823-0048.
[5]Fisher Scientific, Pittsburgh, PA 15219-4785.
[6]Aldrich Chemical Co. Inc., Milwaukee, WI 53233.
[7]Brinkman Instruments Inc., Westbury, NY 11590-0207.
[8]Whatman Inc., Clifton, NJ 07014.

TABLE 1. Percentage recovery of cholesterol oxide standards after washing columns with different solvents[1]

Cholesterol oxides	Solvent IIa	Solvent IIb	Solvent IIc	Solvent IId
	(%)			
6-Ketocholestanol	100[2]	100.0	100	100
7-Ketocholesterol	100	92.08	100	100
22-Ketocholesterol	100	44.96	90.05	100
α-Epoxide	100	98.00	100	100
β-Epoxide	100	59.68	100	100
Cholestanetriol	100	99.14	100	100
7β-OH Cholesterol	100	94.94	100	100
19-OH Cholesterol	100	99.86	100	100
20α-OH Cholesterol	100	36.03	100	100
25-OH Cholesterol	100	88.42	100	100

[1]Solvent IIa = hexane:ethyl acetate = 4:1; Solvent IIb = hexane:ethyl acetate = 1:1; Solvent IIc = hexane:ethyl acetate:ethyl ether = 2:1:1; Solvent IId = hexane:ethyl acetate:ethyl ether = 4:1:2, All vol/vol ratio.

[2]Values are means of four observations.

GC Analysis of Cholesterol Oxides

Analysis of cholesterol oxides was performed with a GC (HP 6890)[9] equipped with an on-column capillary injector and flame ionization detector (FID). A 0.25 mm i.d. × 30 m bonded phase 5% phenylsilicon column with 0.25 μm film thickness (HP-5 column)[9] was used. A splitless inlet was used to inject samples (0.5 μL) into the capillary column, and a ramped oven temperature was used (80 C for 0.25 min, increased to 230 C at 40 C/min, increased to 270 C at 25 C/min, increased to 285 C at 1.5 C/min, and held for 8 min). Temperatures of the inlet and detector were 280 and 280 C, respectively. Helium was the carrier gas at constant pressure of 18.5 psi. Detector (FID) air, H_2, and make-up gas (He) flows were 300, 30, and 28 mL/min, respectively. The area of each peak (pA*sec) was integrated by using ChemStation software[9] and the amount of cholesterol oxides was calculated using an internal standard.

Statistical Analysis

The effect of selected purification solvent systems in meat and egg yolk samples was analyzed independently by SAS® software (SAS Institute, 1988). Analyses of variance were conducted to test the effects of solvent systems on the recovery of cholesterol oxides standards and purification and analysis of egg yolk and meat samples. The Student-Newman-Keuls multiple range test was used to compare differences among mean values. Mean values and SEM are reported.

RESULTS AND DISCUSSION

Data presented in Table 1 show that the recovery of cholesterol oxides standards loaded in silicic column was influenced by the composition of purification solvent. Solvent IIa (hexane:ethyl acetate = 4:1, vol/vol), the most frequently used purification solvent, resulted in 100% cholesterol oxides recovery. However, purifying egg yolk lipids with Solvent IIa was very difficult because of the very high proportions of phospholipids (approximately 30% of total lipids) and cholesterol compared with other animal foods (Stadelman and Cotterill, 1977). Large amounts of cholesterol and phospholipids were still remaining even after washing the silicic acid column with 40 mL of Solvent IIa. By increasing the proportion of polar solvent(s) (Solvent IIb, Solvent IIc, and Solvent IId) the purifying power of Solvent II improved dramatically because of polar characteristics of cholesterol and phospholipids. Except for Solvent IId, however, other solvents (Solvent IIb and Solvent IIc) with high polarity eluted some of the cholesterol oxide standards during purification process (Table 1). Therefore, Solvents IIb and IIc were not appropriate as purification solvent systems, and Solvent IIa (hexane/ethyl acetate = 4:1, vol/vol) and Solvent IId (hexane:ethyl acetate: ethyl ether = 4:1:2, vol/vol/vol) were selected for further comparison using egg yolk lipids and turkey meat lipids.

Data of Table 2 clearly show that the amount of cholesterol oxides in egg yolk powder can vary depending upon the purification solvent systems used. The amount of total cholesterol oxides obtained when Solvent IIa (hexane/ethyl acetate = 4:1, vol/vol) was used was significantly greater than that with the Solvent IId (hexane:ethyl acetate:ethyl ether = 4:1:2, vol/vol/vol). The yields of 7-ketocholesterol and β-epoxide were among the most significantly influenced by the purification solvent. Amounts of other cholesterol oxides, such as α-epoxide, 7α-hydroxycholesterol, 7β-hydroxycholesterol, and total cholesterol oxides, extracted from egg yolk powders were also significantly influenced by the purification solvents but to a lesser degree than those of the 7-ketocholesterol and β-epoxide. The amounts of cholesterol oxides found in turkey leg meat, however, were not affected by the purification solvents.

Cholesterol α- and β-epoxides, 7α- and 7β-hydroxycholesterol, and 7-ketocholesterol were among

[9]Hewlett Packard Co., Wilmington, DE 19808-1610.

TABLE 2. The amount of cholesterol oxides in spray-dried egg yolk powders and cooked meat measured after purification with Solvent IIa and Solvent IId[1]

Cholesterol oxides	Refrigerated yolk[2]			Yolk in ambient temperature[3]			Cooked meat[4]		
	Solvent IIa	Solvent IId	SEM	Solvent IIa	Solvent IId	SEM	Solvent IIa	Solvent IId	SEM
	(μg/cholesterol oxides/g fat)								
7-Ketocholesterol	64.8[a]	31.6[b]	4.7	195.3[a]	105.6[b]	16.8	169.6	149.5	25.3
α-Epoxide	15.9[a]	tr[b]	0.3	34.6[a]	23.3[b]	1.6	21.3	15.6	3.0
β-Epoxide	40.3[a]	19.8[b]	4.1	102.1[a]	52.9[b]	6.3	63.0	34.3	9.8
Cholestanetriol	tr	tr	. . .	60.7[b]	73.1[a]	2.7	. . .	. . .	. . .
7α-OH cholesterol	99.0[a]	50.4[b]	4.9	161.1	180.5	11.0	118.6	106.4	16.0
7β-OH cholesterol	120.6[a]	64.5[b]	6.9	171.5	177.2	11.2	144.8	136.8	24.4
Total cholesterol oxides	340.6[a]	166.5[b]	20.3	725.4[a]	612.5[b]	43.3	517.5	453.5	78.0

[a,b]Means in a row within product with no common superscript differ significantly ($P < 0.05$). Solvent IIa, hexane:ethyl acetate = 4:1 (vol/vol), Solvent IId, hexane:ethyl acetate:ethyl ether = 4:1:2 (vol/vol/vol); tr = trace.

[1]Values are means of four observations.

[2]Stored at 4 C for 8 mo.

[3]Stored at 22 C for 8 mo.

[4]Cooked and stored at 4 C for 7 d.

the most common cholesterol oxides found in egg yolk powder and meat products (Bovenkemp *et al*., 1988; Zubillaga and Maerker, 1991; Engeseth and Gray, 1994; Lai *et al*., 1995b). However, presence of other cholesterol oxides such as 6-ketocholestanol, cholestantriol, 20α-hydroxycholesterol, 25α-hydroxycholesterol, 5,6-hydroxycholesterol, and cholestanediols in meat products and egg powders were also reported (Higley *et al*., 1986; Nourooz-Zadeh and Appelqvist, 1987; Hwang and Maerker, 1993). The content of cholesterol oxides in meat and egg yolk powder varied widely depending upon packaging, heating, irradiation, and storage time and conditions. Freshly prepared egg yolk powder and raw meat reported to have negligible amounts of cholesterol oxides (Pie *et al*., 1991) but the amounts could go up to 1,320 ppm cholesterol oxides for egg yolk powder stored for 7 mo (Morgan and Armstrong, 1987) and 4,844 ppm for cooked bratwurst (Higley *et al*., 1986). Paniangvait *et al*. (1995) indicated that many results reported for cholesterol oxides in foodstuffs, including egg yolk powder and meat, may not be reliable due to variation in methodology such as hot vs cold saponification and choice of analytical tools. Our results in Table 2 indicated that total cholesterol oxides in egg yolk powder (stored for 8 mo at ambient temperature) when purified using the Solvent IId was approximately 300μg/g and that of cooked turkey leg was 32 μg/g. These values were comparable to those of Sander *et al*. (1989), who reported 248 μg cholesterol oxides for commercial egg yolk powders and 21 to 67 μg cholesterol oxides for freeze--dried turkey breast.

Considering 100% recovery for cholesterol oxide standards and the strong purification efficiency of Solvent IId (Table 1), the high cholesterol oxides in egg yolk powders with Solvent IIa (Table 2) should be related to the incomplete purification of cholesterol and phospholipids in egg lipids. Ethyl ether is a good solubilizing agent for neutral lipids, phospholipids, and cholesterol. The addition of ethyl ether to hexane/ethyl acetate increased the polarity of Solvent II and helped the elution of phospholipids and cholesterol while holding cholesterol oxides in silicic column. However, over compensation of the polarity resulted in loss of some cholesterol oxides during the purification step (Table 1). These results suggested that Solvent IId (hexane:ethyl acetate: ethyl ether = 4:1:2, vol/vol/vol) was a superior solvent system as compared with Solvent IIa (hexane/ethyl acetate = 4:1, vol/vol) for the preparation of cholesterol oxides in egg yolk, and these data show that the use of Solvent IId in the purification procedure should result in more accurate measurements of cholesterol oxides in egg yolk samples.

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