Comparison of Solid-Phase Extraction Methods for the Cleanup of Cholesterol Oxidation Products

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Cholesterol oxidation products (COPs) may accumulate in foods of animal origin during processing or storage. As they represent a potential health risk, a need exists for rapid and efficient methods to monitor the occurrence of COPs in the human diet. Methods involving saponification to remove triacylglycerols suffer from artifact formation; therefore, alternatives involving solid-phase extraction (SPE) cartridges have been proposed. The efficiencies of several SPE methods for the cleanup of COPs were compared, and a combination of a silica cartridge followed by an NH_2 cartridge was found to be optimal for the removal of matrix components. COPs added at the 150 ppb level to milk fat were recovered by the method proposed ≥90% except cholestanetriol, for which the recovery rate was only 52%. Separation and quantification of COPs were done by GC/MS in full-scan mode. Splitless injection of COPs at a concentration of 0.3 ng/μL produced signal-to-noise ratios between 1:10 and 1:200 for epoxycholesterol and 7β-hydroxycholesterol, respectively.

Keywords: *Cholesterol oxidation products; oxysterols; solid-phase extraction; milk fat; GC/MS*

INTRODUCTION

Cholesterol is found in most foodstuffs of animal origin in substantial amounts. Like all unsaturated components, it can be attacked by reactive oxygen species, and the resulting products are most commonly termed cholesterol oxidation products (COPs). COPs have been shown to exert a multiplicity of adverse biological effects (Bösinger et al., 1993; Guardiola et al., 1996), and, therefore, nutritionists as well as food scientists are interested in tracking down sources of COPs and in their accurate quantification. Processed (heated, dehydrated, fried) animal foodstuffs (whole milk powder, egg powder, freeze-dried meat, heated animal fats) are the main sources of COPs in the human diet (Paniangvait et al., 1995). Since COPs occur mostly at low levels (parts per billion to parts per million range), they need extensive workup and cleaning procedures before final quantification. A diverse array of sophisticated analytical methodology has been developed for identifying and quantifying COPs. Differences in reported levels for COPs in similar foodstuffs therefore may not only be attributed to differences in manufacturing technology but could also originate from differences in the analytical approach chosen. Capillary gas chromatography coupled to mass spectroscopy is unequivocally accepted as the best means for separating and quantitating COPs with the additional benefit of a simultaneous confirmation of their identity. On the contrary, the preceding sample cleanup methodology is much more heterogeneous. Many authors prefer saponification of either the fat-containing food or the fat extracted as their principal enrichment and cleanup procedure (Finocchiaro et al., 1984; Fischer et al., 1985; Sander et al., 1989; Rose-Sallin et al., 1995), although artifact formation was observed during saponification (Fischer et al., 1985; Maerker and Unruh, 1986). Recently, the negligible destruction of 7-ketocholesterol, the most vulnerable COP to degradation by alkali, during saponification at room temperature was confirmed in a carefully conducted study (Park et al., 1996). Solid-phase extraction (SPE) using either silica (Hwang and Maerker, 1993; Guardiola et al., 1995; Lai et al., 1995), Florisil (Penazzi et al., 1995), aminopropyl (Sallin et al., 1993; Johnson, 1996), or reversed-phase cartridges (Kou and Holmes, 1985; Chen and Chen, 1994) or a combination of these minicolumns (Nourooz-Zadeh, 1990; Nielsen et al., 1995) has been proposed as an alternative to saponification for the isolation and enrichment of COPs. By using deuteriated cholesterol, saponification at room temperature followed by SPE on an aminopropyl column was shown to be a mild and very efficient way to extract and cleanup COPs from milk powder (Rose-Sallin et al., 1995). Schmarr et al. (1996) suggested substituting saponification of the food lipids by transesterification and removal of the bulk of the fatty acid methyl esters formed by silica-SPE. Compared to saponification, analytical approaches involving SPE as the sample preparation technique in COP analysis offer the advantage of being rapid, thus allowing their routine use for the screening of numerous samples, and mild, that is, minimizing artifact formation.

The aim of this study was to compare the efficiencies of different SPE techniques as the sole sample pretreatment before final chromatographic analysis of COPs, starting with a sample amount of 500 mg of milk fat. Sample capacity, purity of the extract, and recovery of COPs from spiked materials were the parameters used for evaluating the merit of the methods compared.

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Table 1. SPE Methods Used for Purification of COPs

SPE cartridge	mobile phase	lipid fraction	ref
C_{18}	(a) 5 mL of ethyl acetate/methanol (1:1)	(a) apolar lipids and cholesterol	Chen and Chen (1994)
	(b) 10 mL of methanol 6 mL of ethyl acetate/methanol (1:1)	(b) COPs	
NH_2	(a) 16 mL of *n*-hexane/ethyl acetate (9:1)	(a) apolar lipids and cholesterol	Bösinger (1991)
	(b) 10 mL of acetone	(b) COPs	
NH_2	(a) 10 mL of *n*-hexane/DCM (9:1) 6 mL of *n*-hexane/DCM (1:1)	(a) apolar lipids and cholesterol	Johnson (1996)
	(b) 5 mL of acetone	(b) COPs	
NH_2[a]	(a) 5 mL of *n*-hexane 5 mL of *n*-hexane/TBME (5:1) 5 mL of *n*-hexane/TBME (3:1)	(a) apolar lipids and cholesterol	Schmarr et al. (1996)
	(b) 5 mL of acetone	(b) COPs	
silica	(a) 3 mL of *n*-hexane/diethyl ether (8:2) 3 mL of *n*-hexane/diethyl ether (1:1)	(a) apolar lipids and cholesterol	Penazzi et al. (1995)
	(b) 3 mL of methanol	(b) COPs	
silica	(a) 10 mL of *n*-hexane/diethyl ether (95:5) 25 mL of *n*-hexane/diethyl ether (90:10) 15 mL of *n*-hexane/diethyl ether (80:20)	(a) apolar lipids and cholesterol	Lai et al. (1995)
	(b) 5 mL of acetone	(b) COPs	
NH_2	(a) 17 mL of *n*-hexane	(a) apolar lipids	Nourooz-Zadeh (1990)
	(b) 25 mL of *n*-hexane/dichloroethane/2-propanol (55:30:15)	(b) cholesterol, cholesterol esters, and COPs	
	solvent removed and residue dissolved in 0.3 mL of acetonitrile:2-propanol (1:1) and applied to C_{18}		
C_{18}	(c) 16 mL of acetonitrile/2-propanol/water (55:25:20)	(c) COPs	

[a] Fat was transesterified with sodium methoxide.

MATERIALS AND METHODS

Materials. To obtain anhydrous milk fat, butter was melted, dried over Na_2SO_4, and filtered. The milk fat used for method comparison was spiked with COPs at a level of 30 μg/g.

Reagents and Chemicals. 5-Cholesten-3β-ol (cholesterol), 5α-cholestane, 5-cholestene-3β,19-diol (19-hydroxycholesterol), 5α,6α-epoxycholesterol, 7-oxocholesten-3β-ol (7-ketocholesterol), 5-cholestene-3β,7β-diol (7β-hydroxycholesterol), 5-cholestene-3β,25-diol (25-hydroxycholesterol), cholestane-3β,5α,6β-triol (cholestanetriol), phosphatidylcholine, and a neutral lipid mixture (cholesterol, cholesteryl oleate, oleic acid, triolein) were purchased from Sigma (St. Louis, MO). Sylon BTF (BSTFA + TMCS, 99:1) was from Supelco (Bellefonte, PA). Methanol, ethyl acetate, diethyl ether, methly *tert*-butyl ether (MTBE), *n*-hexane, dichloromethane (DCM), dichloroethane, acetone, 2-propanol, pyridine, and formic acid were of AR grade (Merck, Darmstadt, Germany). Diethyl ether was freshly distilled before use; other chemicals were used as received.

SPE. Supelclean LC-NH_2 SPE cartridges (500 mg of sorbent) were from Supelco; Sep-Pak Classic cartridges silica (690 mg of sorbent), and C_{18} (360 mg of sorbent) were from Waters (Milford, MA). The SPE methods used for extracting COPs are summarized in Table 1. In all methods 500 mg of milk fat spiked with 30 μg of COPs/g was applied to the columns.

Thin Layer Chromatography (TLC). Precoated Kieselgel 60, 20 × 20 cm, 0.25 mm layer thickness TLC plates were from Merck. The dried residue after SPE was dissolved in 100 μL of DCM, and 10 μL was spotted by means of a microsyringe. The neutral lipid mixture, phosphatidylcholine, and a mixed COP standard were applied as references. Mobile phase was *n*-hexane/diethyl ether/formic acid (80:20:1), and detection was by spraying with 10% H_2SO_4 in methanol and charring at 130 °C.

Silylation. Dried COPs were silylated in screw-capped reaction vials by adding 100 μL of Sylon BTF plus 100 μL of pyridine and heating for 1 h at 80 °C in an oven. After cooling, the sample was dried in a stream of N_2, and the residue was taken up in 50 μL of *n*-heptane.

Gas Chromatography/Mass Spectrometry (GC/MS). One microliter samples were splitless injected at an oven temperature of 100 °C onto a 30 m × 0.32 mm i.d. DB-5 fused silica capillary column (J&W, Folsom, CA), which was operated in a Fisons Instrumens GC 8056 coupled to an MD800 quadrupole mass spectrometer (Fisons Instruments, Milan, Italy). The oven temperature was programmed at 20 °C/min to 280 °C and then at 4 °C/min to 300 °C (held there for 10 min). Helium at 60 kPa was the carrier gas. The temperature of the injector was 300 °C and the transfer time 60 s.

Mass spectra were obtained by using electron impact ionization at 70 eV and a source temperature of 200 °C. The mass range of 100−600 was scanned within 0.8 s. Identification of COPs in milk fat was made by comparison of the retention times and mass spectra to those of authentic standards. Quantification was made by the internal standard method (19-hydroxycholesterol served as internal standard).

RESULTS AND DISCUSSION

Cleanup of COPs. While it is generally recognized that separation, quantification, and identity confirmation of COPs by capillary GC/MS is unsurpassed, different sample preparation and cleanup procedures have been proposed, creating an urgent need for a standardized and validated purification procedure. Food lipids consist of >95% of triaclyglycerols, the remainder being phospolipids, partial glycerides, free fatty acids, sterols, lipid-soluble vitamins, etc. As COPs are present only in trace amounts in the lipid mixture, extensive purification has to be applied to remove the majority of the matrix. The ultimate goal is to increase the absolute amount of COPs in the sample used for the final determination step while reducing matrix components that could compromise chromatographic resolution or interfere with the identification and quantification process during GC or GC/MS.

SPE exploits differences in the polarity of matrix components and the analyte to facilitate separation, with cholesteryl esters being the least polar, phospholipids the most polar, and cholesterol and its oxidation products between. An efficient SPE method should therefore separate COPs from the bulk of the triacylglycerols present, with a minimum of contamination in the COPs-containing fraction by coeluted cholesterol. We compared the efficiencies of seven SPE procedures

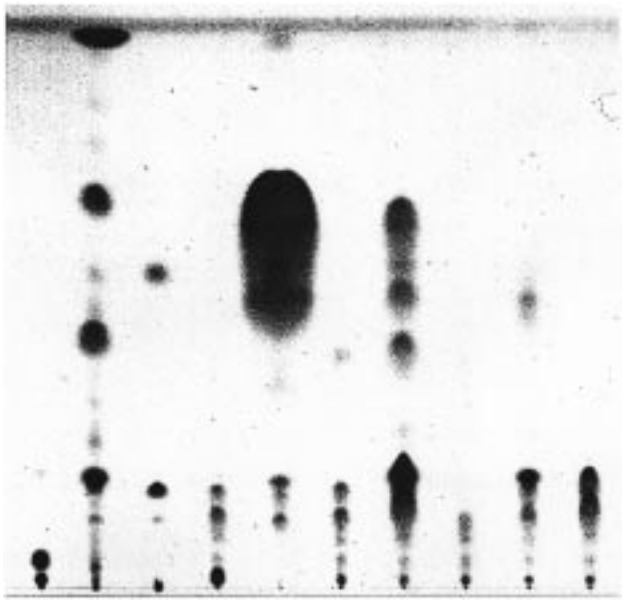

Figure 1. Efficiency of several SPE procedures for the purification of COPs: lane 1, COPs standard solution; lane 2, lipid standard (cholesteryl oleate, triolein, oleic acid, and cholesterol); lane 3, phosphatidylcholine; lane 4, silica-SPE (Schmarr et al., 1996); lane 5, C_{18}-SPE (Chen and Chen, 1994); lane 6, NH_2-SPE (Bösinger, 1991); lane 7, silica-SPE (Penazzi et al., 1995); lane 8, silica-SPE (Lai et al., 1995); lane 9, NH_2- + C_{18}-SPE (Nourooz-Zadeh, 1992); lane 10, NH_2-SPE (Johnson, 1995).

for the purification of COPs found in the literature. All procedures were devised for sample loads of 200 mg up to 1 g of milk fat, lard, or fat extracted from fresh or dehydrated chicken egg. Bovine milk fat (500 mg) spiked with 30 μg/g 19-hydroxycholesterol, 7-ketocholesterol, 7β-hydroxycholesterol, α-epoxycholesterol, 25-hydroxycholesterol, and cholestanetriol served as a model substrate. Only the SPE separation step was performed; any other additional purification procedure, as foreseen in some of the methods, was deliberately omitted. It was our intention to keep the purification as simple as possible to minimize artifact formation. The purified extracts were separated by TLC and visualized by charring to allow comparison of the purity of the individual extracts (Figure 1).

The SPE cleanup procedure using a C_{18} cartridge (lane 5) as described by Chen and Chen (1994) and the procedure according to Penazzi et al. (1995) involving a silica cartridge (lane 7) proved to be least efficient in removing matrix material. Both methods were originally intended to be used in connection with HPLC for the final separation and quantification of COPs. Using a silica-SPE cartridge and the elution scheme devised by Lai et al. (1995) produced the cleanest extract (lane 8). Nevertheless, spurious peaks were seen in the total ion chromatogram (TIC) when the silylated extract was separated by GC/MS.

The remaining procedures (lanes 4, 6, 9, and 10), although efficient in removing the bulk of triacylglycerol, left traces of cholesterol and/or partial glycerides in the extract. In the cleanup procedure described by Nourooz-Zadeh (1992) (lane 9), in addition to SPE, an HPLC purification stage before final GC quantification is foreseen, and in the Johnson (1995) method (lane 10) the SPE is followed by a transmethylation and a saponification step. Therefore, both approaches include an extensive and time-consuming sample preparation sequence.

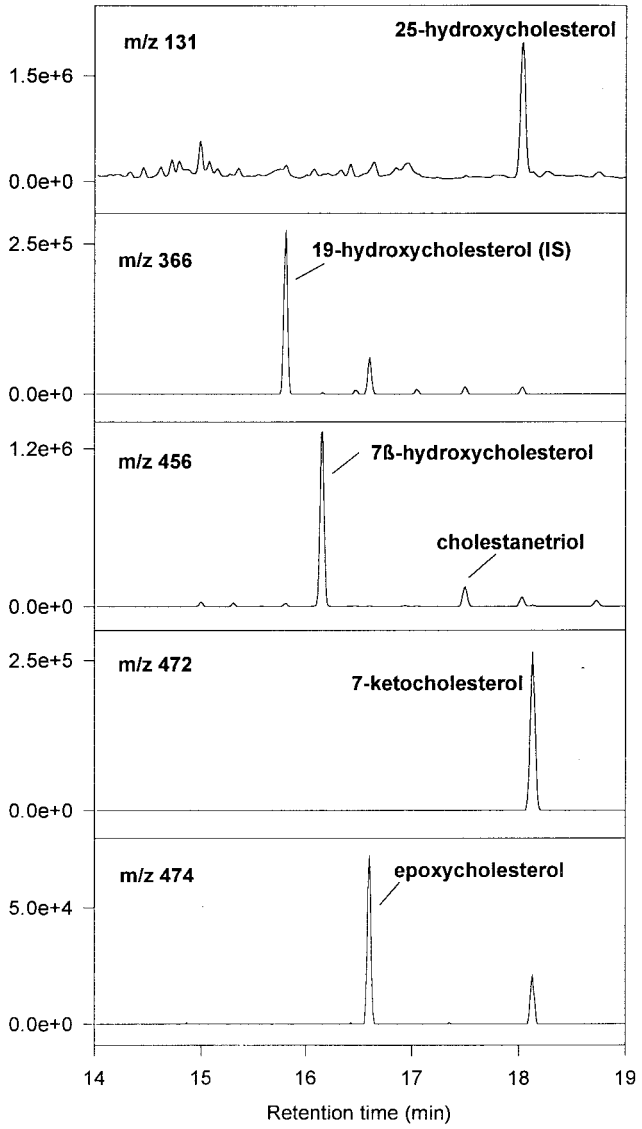

Figure 2. Mass chromatograms used for the quantification of COPs isolated from milk fat spiked with 1200 ng/g of indivdual COPs.

To reduce contaminants to a further degree, the COPs-containing eluate of the silica-SPE method according to Lai et al. (1995) was taken up in 1 mL of *n*-hexane/ethyl acetate (9:1) and applied to an NH_2-SPE cartridge, which was eluted with a further 15 mL of the same mobile phase (Bösinger, 1991). Purified COPs were eluted with 10 mL of acetone. The combined procedure gave very clean mass chromatograms, as demonstrated in Figure 2 by GC/MS of TMS derivatives of COPs extracted from milk fat spiked at the 1 200 ng/g level.

GC/MS Quantification of COPs. Characteristic ions used for monitoring and quantifying COPs are summarized in Table 2. All of these diagnostic ions have been described previously as being useful for the identification and quantification of COPs (Rose-Sallin et al., 1995; Lai et al., 1995). We did not use the molecular ion in each case for the quantification of the COPs, as suggested by Nielsen et al. (1995), to increase sensitivity. Since the MS detector was run in full-scan mode, the identities of the targets were verified by a spectral match against authentic standards. Quantification of COPs over the range 0.3−12 ng was done by using 19-hydroxycholesterol as internal standard. Re-

Table 2. Performance Criteria for the Mass Spectrometric Quantification of COPs

TMS deriv	ion used for quantn (*m*/*z*)	signal-to-noise ratio at 0.3 ng	recovery (%) at 150 ng/g	recovery (%) at 1200 ng/g
7β-hydroxycholesterol	456	1:200	97	98
epoxycholesterol	384	1:10	– [a]	101
cholestanetriol	403	1:60	52	56
25-hydroxycholesterol	131	1:50	97	97
7-ketocholesterol	472	1:30	92	104
19-hydroxycholesterol	353	–		

[a] Not detected.

gression analysis of the plots of the peak area ratio versus the mass ratio was linear over the concentration range considered. The correlation coefficient of the calibration equations was used for judgment of linearity, and they were 0.998 at least. The signal-to-noise ratios for the calibration solution containing the COP standards at the lowest concentration, that is, 0.3 ng/μL, are also listed in Table 2. Rose-Sallin et al. (1995) and Nielsen et al. (1995) found lower limits of detection (0.0003−0.035 ng/μL, depending on the nature of the COP), but used selected ion monitoring, whereas in this study the MS detector was operated in the full-scan mode, allowing an unequivocal identification of compounds. Repeatability of the GC/MS quantification method, as judged by the relative standard deviation of six repeated injections of a solution containing COPs at 6 ng/μL, was 0.65−3.70%.

Recovery of COPs added to milk fat at concentrations of 150 and 1200 ng/g varied between 52 and 104% (Table 2). These recovery rates refer to the internal standard (19-hydroxycholesterol) and are, therefore, relative figures. Substantial amounts of cholestanetriol, the most polar COP studied, were lost during cleanup; the remaining COPs were quantitatively recovered, irrespective of the spike level. Lai et al. (1995) as well as Nielsen et al. (1995) reported for their procedures (silica-SPE, NH_2-SPE) a recovery rate of 86% for cholestanetriol, whereas Chen and Chen (1995) found a lower recovery rate of 70% using silica-SPE. Checking the recovery rates for the two SPE steps involved in our procedure individually showed that the loss of cholestanetriol occurred during NH_2-SPE. As cholestanetriol is a COP that is only rarely found in processed foodstuffs containing cholesterol (Paniangvait et al., 1995), no further experiments were conducted to improve its recovery.

Conclusion. SPE extraction procedures devised for the cleanup of COPs differ largely in their ability to reduce matrix background. A combination of silica-SPE and NH_2 cartridges eliminated to a large extent fatty material from the analytes and allowed a reliable separation and quantification of COPs.

LITERATURE CITED

Bösinger, S. Zum Vorkommen von Cholesterinoxidationsprodukten in Milchprodukten. Ph.D. Dissertation, Veterinärmedizinische Universität, Wien, 1991.

Bösinger, S.; Luf, W.; Brandl, E. Oxysterols: Their occurrence and biological effects. *Int. Dairy J.* **1993**, *3*, 1−33.

Chen, B. H.; Chen, Y. C. Evaluation of the analysis of cholesterol oxides by liquid chromatography. *J. Chromatogr. A* **1994**, *661*, 127−136.

Finocchiaro, E. T.; Lee, K.; Richardson, T. Identification and quantification of cholesterol oxides in grated cheese and bleached butteroil. *J. Am. Oil Chem. Soc.* **1984**, *61*, 877−883.

Fischer, K.-H.; Laskawy, G.; Grosch, W. Quantitative Analyse von Autoxidationsprodukten des Cholesterols in tierischen Lebensmitteln (Quantitative analysis of cholesterol autoxidation products in animal foods). *Z. Lebensm. Unters. Forsch.* **1985**, *181*, 14−19.

Guardiola, F.; Codony, R.; Rafecas, M.; Boatella, J. Comparison of three methods for the determination of oxysterols in spray-dried egg. *J. Chromatogr. A* **1995**, *705*, 289−304.

Guardiola, F.; Codony, R.; Addis, P. B.; Rafecas, M.; Boatella, J. Biological effects of oxysterols: Current status. *Food Chem. Toxicol.* **1996**, *34*, 193-211.

Hwang, K. T.; Maerker, G. Quantitation of cholesterol oxidation products in unirradiated and irradiated meats. *J. Am. Oil Chem. Soc.* **1993**, *70*, 371−375.

Johnson, C. B. Isolation of cholesterol oxidation products from animal fat using aminopropyl solid-phase extraction. *J. Chromatogr. A* **1996**, *736*, 205−210.

Kou, I. L.; Holmes, R. S. The analysis of 25-hydroxycholesterol in plasma and cholesterol-containing foods by high-performance liquid chromatography. *J. Chromatogr.* **1985**, *330*, 339−346.

Lai, S.-M.; Gray, J. I.; Zabik, M. E. Evaluation of solid-phase extraction and gas chromatography for determination of cholesterol oxidation products in spray-dried whole egg. *J. Agric. Food Chem.* **1995**, *43*, 1122−1126.

Maerker, G.; Unruh, J. Cholesterol oxides I. Isolation and determination of some cholesterol oxidation products. *J. Am. Oil Chem. Soc.* **1986**, *63*, 767−771.

Nielsen, J. H.; Olsen, C. E.; Duedahl, Ch.; Skibsted, L. H. Isolation and quantification of cholesterol oxides in dairy products by selected ion monitoring mass spectrometry. *J. Dairy Res.* **1995**, *62*, 101−113.

Nourooz-Zadeh, J. Determination of the autoxidation products from free or total cholesterol: A new multistep enrichment methodology including the enzymatic release of esterified cholesterol. *J. Agric. Food Chem.* **1990**, *38*, 1667−1673.

Paniangvait, P.; King, A. J.; Jones, A. D.; German, B. G. Cholesterol oxides in foods of animal origin. *J. Food Sci.* **1995**, *60*, 1159−1174.

Park, P. W.; Guardiola, F.; Park, S. H.; Addis, P. B. Kinetic evaluation of 3β-hydroxycholest-5-en-7-one (7-ketocholesterol) stability during saponification. *J. Am. Oil Chem. Soc.* **1996**, *73*, 623−629.

Penazzi, G.; Caboni, M. F.; Zunin, P.; Evangelisti, F.; Tiscornia, E.; Gallina Toschi, T.; Lercker, G. Routine high-performance liquid chromatographic determination of free 7-ketocholesterol in some foods by two different analytical methods. *J. Am. Oil Chem. Soc.* **1995**, *72*, 1523−1527.

Rose-Sallin, Ch.; Huggett, A. C.; Bosset, J. O.; Tabacchi, R.; Fay, L. B. Quantification of cholesterol oxidation products in milk powders using [$^2H_7$]cholesterol to monitor cholesterol autoxidation artifacts. *J. Agric. Food Chem.* **1995**, *43*, 935−941.

Sallin, Ch.; Baumann, E.; Bütikofer, U.; Sieber, R.; Bosset, J. O. Contribution to oxidized cholesterol determination in milk and milk products. I. Possibilities and limits of RP-HPLC techniques. *Mitt. Geb. Lebensmittelunters. Hyg.* **1993**, *84*, 141−157.

Sander, B. D.; Addis, P. B.; Park, S. W.; Smith, D. E. Quantification of cholesterol oxidation products in a variety of foods. *J. Food Prot.* **1989**, *52*, 109−114.

Schmarr, H.-G.; Gross, H. B.; Shibamoto, T. Analysis of polar cholesterol oxidation products: Evaluation of a new method involving transesterification, solid-phase extraction, and gas chromatography. *J. Agric. Food Chem.* **1996**, *44*, 512−517.

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