Analysis of Polar Cholesterol Oxidation Products: Evaluation of a New Method Involving Transesterification, Solid Phase Extraction, and Gas Chromatography

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Monitoring cholesterol oxidation products is important in the evaluation of the potential health risks associated with lipid oxidation. In the present study, a method allowing quick and reliable analysis of polar cholesterol oxidation products was evaluated. After Soxhlet-lipid extraction, the fat was transesterified under mild conditions, thereby minimizing degradation and allowing determination of the free and esterified cholesterol oxides. Sample fractionation was achieved with aminopropyl solid phase extraction cartridges and a stepwise elution with hexane, hexane/methyl *tert*-butyl ether, and acetone to separate polar cholesterol oxides from cholesterol and other lipid products. Further analysis of the trimethylsilyl derivatives was performed by gas chromatography with detection by flame ionization or mass spectrometry. A phytosterol oxide such as sitosterol α-epoxide (24α-ethyl-5α,6α-epoxycholestan-3β-ol) was employed for the first time as an internal standard for the quantification of cholesterol oxides in foodstuffs of animal origin.

Keywords: *Cholesterol oxides; oxysterols; solid phase extraction; amino phase; gas chromatography*

INTRODUCTION

Cholesterol, as the major sterol in human and animal tissue, undergoes autoxidation under various conditions yielding a large variety of oxidation products (oxysterols) (Smith, 1981). The demonstration that some cholesterol oxides possess biological activity, such as cytotoxicity (Higley and Taylor, 1984; Naseem and Heald, 1987), mutagenicity (Sevanian and Peterson, 1986), angiotoxicity (Dorset, 1992; Peng et al., 1991), or carcinogenicity (Morin et al., 1991), together with their discovery in foodstuffs and human tissues has attracted much interest both in the food science and medical fields. Furthermore, cholesterol oxidation products seem to play an important role in the development of human atherosclerosis (Imai et al., 1976; Morin and Peng, 1989; Peng et al., 1990, 1991; Hodis et al., 1991; Naresh and Singhal, 1991). Reviews of the biological effects of cholesterol oxidation products were published recently (Peng and Morin, 1992) and (Boesinger et al., 1993). Due to their potential health risk, the formation and presence of oxysterols in foods have been the subject of many studies (Chicoye et al., 1968; Finocchiaro and Richardson, 1983; Tsai and Hudson, 1984; Missler et al., 1985; Park and Addis, 1986; Higley et al., 1986; Nourooz-Zadeh and Appelqvist, 1987, 1988, 1989; Sander et al., 1989; Yan and White, 1990; Ohshima et al., 1993).

Various methods have been developed for the analysis of cholesterol oxidation products, from early thin-layer chromatography (TLC) to high-performance liquid chromatography (HPLC) or capillary gas chromatography (GC). Even a nuclear magnetic resonance technique (NMR) was recently employed (Fontana et al., 1992, 1993). Most often, chromatographic techniques are applied to separate the various compounds not only from each other but also from the large amounts of interfering triglycerides, cholesterol, phospholipids, and other matrix lipids present in the sample. A combination of liquid column chromatography (LC) or preparative TLC prior to HPLC or GC is often used to achieve good sample fractionation and concentration of the cholesterol oxides (Fischer et al., 1985; Bovenkamp et al., 1988; Pie et al., 1990).

However, reliable routine analysis of cholesterol oxidation products is still a difficult and lengthy procedure. The goal of this study was to develop a modified method for fast analysis of cholesterol oxides, allowing routine screening of numerous samples. Sample preparation, GC analysis, method evaluation, and application to some foodstuffs potentially containing cholesterol oxidation products due to production processes or storage conditions are described.

MATERIALS AND METHODS

Reagents. Cholesterol (cholest-5-en-3β-ol), 7α- and 7β-hydroxycholesterol (5-cholestene-3β,7α-diol and 5-cholestene-3β,7β-diol), 19-hydroxycholesterol (5-cholestene-3β,19-diol), 20α-hydroxycholesterol (5-cholestene-3β,20α-diol), 25-hydroxycholesterol (5-cholestene-3β,25-diol), cholesterol α-epoxide (5α,6α-epoxycholestan-3β-ol), 7-ketocholesterol (7-oxo-5-cholesten-3β-ol) and cholestane-3β,5α,6β-triol were obtained from Sigma Chemical Company, St. Louis, MO. Cholesterol β-epoxide (5β,6β-epoxycholestan-3β-ol) and 3β,5-dihydroxy-5α-cholestan-6-one (6-oxo-cholestane-3β,5α-diol) were obtained from Steraloids Inc., Wilton, NH. Sodium methylate (1 M in methanol), sitosterol, stigmasterol, *m*-chloroperbenzoic acid, methyl *tert*-butyl ether (MTBE), and dry pyridine were purchased from Fluka (Buchs, Switzerland). *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) was obtained from Regis Chemical Company or Fluka. Other reagent grade chemicals were purchased from Fisher Scientific Company, Medford, MA. Solid phase extraction (SPE) cartridges (Bondelut, amino-phase) were obtained from Varian, Walnut Creek, CA.

Synthesis of the internal standard, sitosterol α-epoxide (24α-ethyl-5α,6α-epoxycholestan-3β-ol) was performed according to a procedure described by Fieser and Fieser (1967) for the

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homologous cholesterol derivative. Briefly, purified, recrystallized sitosterol was epoxidated with *m*-chloroperbenzoic acid in dry dichloromethane as reagent and the crude mixture was purified via LC on silica gel. After recrystallization from methanol, purity and identity was checked by TLC and GC-MS. The highest signal in the mass spectrum of the trimethylsilyl ether derivative of the synthesized sitosterol α-epoxide was at m/z 502, assigned as the molecular ion [M^+]. Other signals were at 487 [M − CH_3], 484 [M − H_2O], 473 [M − C_2H_5], 431 [M − $CH_3CHOHCHCH_2$], 412 [M − TMSiOH], 397 [M − CH_3 − TMSiOH], 394 [M − H_2O − TMSiOH], and 368 [M − CH_3 − C_2H_5 − TMSiOH]. The fragmentation was in accordance with data published by Nourooz-Zadeh and Appelqvist (1992) for the sitosterol α-epoxide and data published by Annan (1993) for the homologous cholesterol epoxide.

Lipid Extraction. Samples were homogenized in a mortar and then transferred into thimbles for continuous liquid extraction with a Soxhlet apparatus. Pasty samples were mixed with anhydrous sodium sulfate and purified sand for better extraction. The extraction proceeded for 6−8 h using MTBE as solvent. To prevent potential oxidation due to light sources, the glassware was wrapped with aluminum foil. After evaporation of the solvent with a rotary evaporator, the lipid extracts were dried in a vacuum desiccator and stored under nitrogen in a sealed vial in the refrigerator (5 °C) until further analysis (storage time, 1−4 weeks).

Transesterification. First, 150−220 mg of fat was weighed in a 12-mL conical centrifuge vial with tightly fitting screw cap or ground joint stopper. Then, 5 μL of a 1-μg/μL solution of the internal standard in MTBE was added, representing ∼20−40 ppm in the fat. For transesterification, 2 mL of 10% sodium methylate in methanol, diluted with MTBE (4:6, v/v), was added and mixed by vortex for 1 min. The mixture was allowed to stand for 1 h at room temperature with additional mixing after ∼30 min. Then, 2 mL of water and 5 mL of chloroform were added and the vial was closed with a tight cap. To extract the organic material, the sample was shaken for 1 min. After 5 min of centrifugation at 2000 rpm to facilitate phase separation, the upper (aqueous) phase was removed with a Pasteur pipet. Neutralization of excessive alkali was accomplished by adding 2 mL of 1% citric acid in water, shaking, centrifuging, and again disposure of the aqueous phase. The residing chloroform extract was then evaporated under a stream of nitrogen in a warm water bath with an analytical evaporator (Organomation Associates, Inc., Northborough, MA).

LC Fractionation on Amino-Phase SPE Cartridges. To bind traces of moisture and to ensure reproducible retention behavior, a small amount of anhydrous sodium sulfate was placed on top of 500 mg of bonded amino-phase SPE cartridges that were then conditioned with 5 mL of hexane. The transesterified lipid was redissolved with 250 μL of chloroform, then transferred onto the cartridge with a Pasteur pipet, and then washed again with 2 × 2.5 mL of hexane. Apolar material and free cholesterol were then eluted with another 5 mL of hexane, 5 mL of hexane/MTBE (5:1, v/v) and 5 mL of hexane/MTBE (3:1, v/v). A polar fraction containing the components of interest was eluted with 7 mL of acetone and collected in conical centrifuge vials with ground joint stoppers, easing further sample treatment. Elution from the SPE cartridge proceeded under gravity only. A stainless steel Luer-tip needle attached to the tip of the cartridge yielded a flow rate of 0.3−0.5 mL/min.

Derivatization of the Sterol Oxides to Trimethylsilyl Ethers (TMS Ethers). The oxysterol-containing acetone fraction was dried under a stream of nitrogen. Then, 100 μL of dry pyridine and 100 μL of BSTFA + 1% TMCS were added and, after flushing with nitrogen, the vial was sealed with a glass stopper. Gently turning the vial and final vortexing ensured good solvation of the residue in the reagent. After a reaction time of 3−4 h at room temperature, the sample was ready for injection into the gas chromatograph.

Capillary Gas Chromatography with Flame Ionization Detection (GC-FID). The TMS derivatives of the cholesterol oxides were analyzed by capillary GC on a HP 5890 (Hewlett-Packard, Palo Alto, CA) equipped with a flame ionization detector. To lower the elution temperature of the relatively apolar TMS ethers of the cholesterol oxides to reduce potential degradation of labile components, a polar and high-temperature-stable stationary phase was used. A phenylmethyl polysiloxane (50% phenyl) was coated in the laboratory onto phenyl-dimethyl deactivated fused silica (Polymicro Technologies, Phoenix, AZ), following standard procedures for column preparation as described earlier (Grob, 1986). The stationary phase, SOP-50, was obtained from BGB-Analytik (Adliswil, Switzerland). The column system for the analysis consisted of a 1 m × 0.32 mm i.d. phenyldimethyl deactivated uncoated precolumn (retention gap) coupled via a press-tight connector (Restek Corporation, Bellefonte, PA) to a 25 m × 0.25 mm i.d. analytical column coated with a 0.15-μm film of SOP-50. Splitless injection (0.5 min splitless time) was performed at 290 °C, and column temperature was programmed from 110 °C (2 min hold) to 235 °C (5 min hold) at 40 °C/min and then to 320 °C (5 min hold) at 3.0 °C/min. Detector temperature was held at 330 °C. Helium was used as the carrier gas at a constant inlet pressure of 140 kPa. A GC-FID chromatogram of the standard mixture is shown in Figure 1, which indicates good resolution and low bleed even at elevated temperatures.

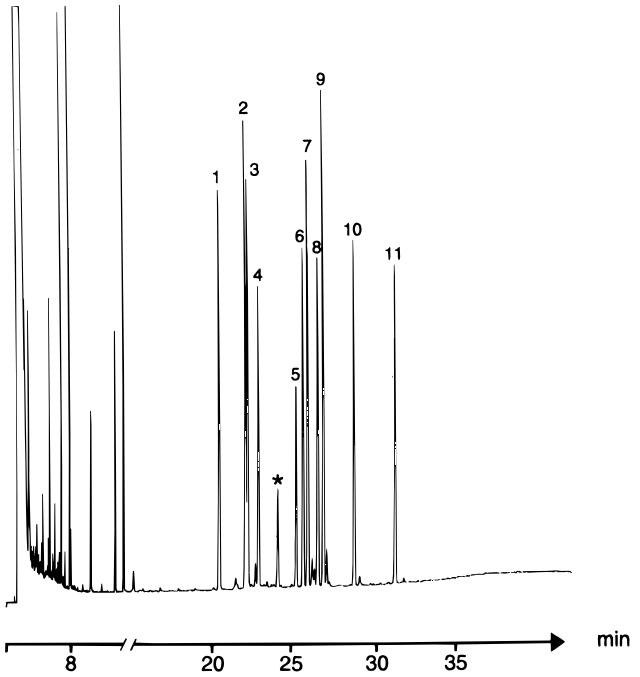

Figure 1. GC-FID chromatogram of standards (TMS ethers): (**1**) 7α-hydroxycholesterol; (**2**) 19-hydroxycholesterol; (**3**) cholesterol; (**4**) 7β-hydroxycholesterol; (**5**) cholesterol β-epoxide; (**6**) cholesterol α-epoxide; (**7**) cholestane-3β,5α,6β-triol; (**8**) 25-hydroxycholesterol; (**9**) 20α-hydroxycholesterol; (**1**), 7-keto-cholesterol; (**11**) 3β,5-dihydroxy-5α-cholestan-6-one; (*****) secondary derivatization product of 20α-hydroxycholesterol (see also footnote in Table 1). GC conditions as described under Materials and Methods.

Gas Chromatography−Mass Spectrometry (GC-MS). Verification of peak assignment was supported by GC-MS on a HP 5890 Series II GC with a MSD 5971 as the mass selective detector. The mass spectrometer interface temperature was set to 320 °C, and the electron energy was 70 eV. The column used for GC-MS was a 30 m × 0.25 mm i.d. DB-5MS fused silica capillary with a film thickness of 0.25 μm (Fisons-J&W, Folsom, CA). Splitless-injection (0.5 min splitless-time) was performed at 290 °C, and column temperature was programmed from 110 °C (2 min hold) to 235 °C (5 min hold) at 40 °C/min and then to 310 °C (5 min hold) at 3.0 °C/min. Helium was used as carrier gas at a constant inlet pressure of 145 kPa without vacuum compensation.

Recoveries. To evaluate the recoveries of the different oxides, 100−150 mg of coconut oil (containing virtually no cholesterol) was spiked with 50 μL of a 100 ng/μL standard solution containing cholesterol and the cholesterol oxides. The

Table 1. Recoveries of Oxysterols Obtained by Transesterification Method

oxysterol	mean, %	standard deviation, %	variance, %
7α-hydroxycholesterol	97.2	2.1	2.2
7β-hydroxycholesterol	95.9	1.4	1.5
19-hydroxycholesterol	86.9	2.2	2.5
20α-hydroxycholesterol[a]	100.6	2.6	2.5
25-hydroxycholesterol	97.2	1.1	1.1
cholesterol α-epoxide	100.2	2.1	2.1
cholesterol β-epoxide	107.0	3.4	3.2
7-ketocholesterol	94.1	1.4	1.5
3β,5-dihydroxy-5α-cholestan-6-one	98.8	4.0	4.1
cholestane-3β,5α,6β-triol	85.8	2.5	2.9
cholesterol	1.2	0.4	35.3

[a] Derivatization of 20α-hydroxycholesterol yields two products that are fully converted to one only after a reaction period of ~1 day. Therefore, quantitation was performed with the sum of both products.

samples were taken through the entire procedure. After evaporation of the cholesterol oxide containing acetone fraction, 5 μL of the internal standard solution (in this case stigmasterol at 1 μg/μL) and 200 μL of the derivatization agent were added. Chromatographic analysis was performed by GC-FID on a SOP-50 column. Recoveries were calculated with the relative response factors.

RESULTS

Method Evaluation. The data obtained from the recovery test (Table 1) show good results for both moderate polar (epoxides) and polar oxysterols. Even the most polar oxysterol, the cholestane-3β,5α,6β-triol, is recovered satisfactorily at >85%. The low recovery of cholesterol indicates a sufficient separation of the oxysterol containing fraction from the free sterol. Furthermore, the good recovery and low standard deviation values show that there is no obvious damage or loss to critical compounds, such as the epoxides or the 7-ketocholesterol, during sample preparation.

Application to Foodstuffs. The applicability of this new method is demonstrated by the analysis of several foodstuffs of animal origin for the presence of oxysterols. The GC-FID chromatogram of the oxysterol-containing fraction of a cheese-spread is shown in Figure 2 as an example. Detection of several oxysterols is in the low ppm range (mg/kg fat). Therefore, one works close to the detection limit of this method (0.1−0.5 ppm), and verification of peak assignments by GC-MS is necessary.

The results for some foodstuffs are given in Table 2. In general, the amounts of oxysterols are low (<0.5−3 ppm) as was found by other authors [*e.g.*, Hwang and Maerker (1993)]. The exception in our study was the liverwurst, which had up to five times higher concentrations of oxysterols compared with other samples. One possible explanation could be the higher metabolism rate in the liver tissue; however, we did not study that case further.

DISCUSSION

Method Development. To develop an applicable method, the following difficulties were considered: (1) most oxidation products are present at a low ppm or ppb level; (2) their polarities vary from apolar (*e.g.*, cholest-4-en-3-one) to very polar (*e.g.*, cholestane-3β,5α,6β-triol); (3) oxidation products may be present in free or in esterified form; (4) components are likely to oxidize or otherwise degrade during sample preparation (*e.g.*, 7-ketocholesterol or the epoxides), thus calling for mild extraction and sample preparation procedures as well as inert chromatography conditions; (5) an internal standard is required for reliable quantification; (6) peak assignment verification should be supported by a MS method providing additional information about peak purity.

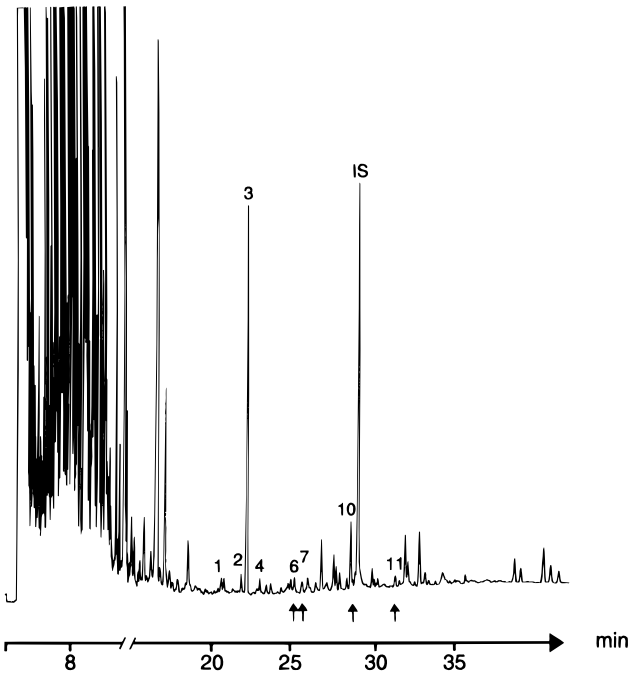

Figure 2. GC-FID chromatogram of the oxysterol containing fraction from a cheese spread. Peak assignment as in Figure 1. Internal standard (IS) sitosterol α-epoxide. GC conditions as described under Materials and Methods.

Lipid Extraction. A popular method for total lipid extraction is the procedure of Folch (Folch et al., 1957) in which samples are homogenized in a mixture of chloroform and methanol as the extracting solvents. However, in many cases, awkward emulsions are formed, rendering the extraction procedure time-consuming and precise phase separation difficult. Therefore, a continuous liquid extraction with a Soxhlet-apparatus was employed. As an extraction solvent, MTBE was preferred over diethyl ether because MTBE contains no stabilizers or peroxides (Little et al., 1979). A polar solvent must be chosen to increase recovery of otherwise poorly soluble polar cholesterol oxides, such as cholestane-3β,5α,6β-triol. Potential oxidation of lipids was further minimized by wrapping the Soxhlet apparatus with aluminum foil, thus preventing exposure to light sources.

Saponification versus Transesterification. Hot or cold saponification has often been used as an enrichment step in the quantitative determination of cholesterol or its oxidation products. It serves not only to remove the triglycerides but also to saponify esterified cholesterol oxides, thus allowing determination of free and esterified oxysterols. However, the drawback of hot saponification is the potential loss of labile compounds, such as the 5,6-epoxycholestanols (Tsai et al., 1980), whereas cold saponification shows no artifact formation (Park and Addis, 1986) but requires long reaction times. A milder, enzymatic procedure for the release of esterified cholesterol oxides was introduced by Nourooz-Zadeh (1990). However, that procedure involves several enrichment steps by TLC and solid-phase extraction, and therefore is time-consuming and hardly applicable for routine analysis. Some authors describe methods with

Table 2. Quantification of Oxysterols in Foodstuffs[a]

sample	7α-hydroxy-cholesterol	7β-hydroxy-cholesterol	19-hydroxy-cholesterol[b]	20α-hydroxy-cholesterol	25-hydroxy-cholesterol	cholesterol α-epoxide	cholesterol β-epoxide	7-keto-cholesterol	3β,5-dihydroxy-5α-cholestan-6-one	cholestane-3β,5α,6β-triol
salame (A)	2.21 ± 0.42	0.88 ± 0.07	ND	0.34 ± 0.03	ND	0.74 ± 0.19	0.50 ± 0.04	1.09 ± 0.01	ND	ND
salame (B)	0.38 ± 0.08	0.36 ± 0.13	ND	ND	ND	ND	0.35 ± 0.04	0.57 ± 0.01	ND	ND
parmesan	1.16 ± 0.05	1.31 ± 0.04	0.31 ± 0.03	2.81 ± 0.27	0.57 ± 0.05	0.93 ± 0.08	0.71 ± 0.21	1.22 ± 0.01	ND	ND
cheese spread	0.59 ± 0.02	0.75 ± 0.01	0.74 ± 0.02	ND	0.51 ± 0.02	0.68 ± 0.04	0.71 ± 0.03	0.48 ± 0.03	0.44 ± 0.04	ND
pork links	0.55 ± 0.19	1.50 ± 0.17	ND	ND	ND	2.14 ± 0.39	0.69 ± 0.22	3.84 ± 0.11	ND	ND
liverwurst	0.86 ± 0.14	1.15 ± 0.09	ND	10.88 ± 1.14	1.55 ± 0.30	3.75 ± 1.46	1.44 ± 0.22	1.67 ± 0.40	2.34 ± 0.88	9.27 ± 2.22

[a] Results are expressed in milligrams per kilogram of fat, extracted from the corresponding foodstuff; mean ± SD (n = 3); ND, not detected. [b] Peak identification is tentative because of interfering compounds.

only chromatographic separation of the polar oxysterols from interfering apolar lipids without prior liberation of esterified cholesterol oxides (Zubillaga and Maerker, 1984, 1991; Nourooz-Zadeh and Appelqvist, 1987; Sallin et al., 1993; Chen and Chen, 1994). In this case, depending on the samples analyzed, only a fraction of existing oxysterols will be recovered.

Besides the disadvantage of possible artifact formation, saponification also has some practical drawbacks. The saponified triglycerides form a soap solution that renders extraction of the unsaponifiable material tedious because of the bad separation of the evolved emulsions. Micelle formation, thus loss of compounds of interest, is another known side-effect when extracting from detergent-containing solutions (Lognay et al., 1989).

As a means of liberating alcohols, transesterification is known to be a good alternative to saponification. It was succesfully used and described in detail in a recent method for the determination of the sum of free and esterified sterols in the analysis of edible oils by an on-line LC-GC method (Biedermann et al., 1993). The conditions for transesterification are mild compared with saponification, and triglycerides are converted to fatty acid methyl esters within minutes at room temperature (sterol esters need a slightly longer period of (1−2 h; Zubillaga and Maerker, 1988). Zubillaga and Maeker also demonstrated that no artifacts were formed during the reaction.

Considering these benefits, transesterification was our method of choice for both liberating bound (esterified) oxysterols as well as converting triglycerides into fatty acid methyl esters. The whole reaction and subsequent extraction was easily accomplished in a centrifuge vial, which restricted the use of glassware and, therefore, also minimizing the loss of substances due to adsorption or during the transfer of liquids. The reaction time of 1 h proved to be sufficient and yielded reproducible results. Sodium methylate (in methanol) with MTBE as solvent to mix the lipids into the methanolic solution was chosen according to Biedermann.

LC Separation. The solubility of the most polar compound of interest (cholestane-3β,5α,6β-triol) and the retaining capacity of the stationary phase were considered in the decision to transfer the dried chloroform extract onto the SPE cartridges. Dissolving the extract in 250 μL of chloroform followed by two vial rinses with 2.5 mL of hexane proved to be sufficient for complete recovery without excessive deactivation of the sorbent and without the loss of intermediate polar components such as the 5,6-epoxides.

After transesterification, the lipid primarily consists of fatty acid methyl esters, free cholesterol and its oxidation products, and some minor apolar as well as polar components. As we were only interested in cholesterol oxidation products that are more polar than the cholesterol itself, the following LC procedure had to be optimized to elute apolar components up to the polarity of the free cholesterol. This optimization was achieved by the stepwise procedure described in the methods and final elution of the polar fraction that contains the components of interest.

In the early stages of this method development, silica gels of various origin and particle sizes were tested for their usefulness as sorbents in either commercially available or self-prepared (glass column) cartridges. However, to elute the relatively polar cholestane-

3β,5α,6β-triol, methanol had to be used as eluent. Unfortunately, methanol also elutes (or partly dissolves) silica gel particles, which is referred to as "fine" by the commercial suppliers of packing materials. Because this "fine" caused problems when working with syringe needles of small bores or introduced adsorptive material into the GC apparatus with on-column injection, we finally switched to aminopropyl solid-phase extraction cartridges. Using an amino phase, the cholestane-3β,5α,6β-triol could be quantitatively eluted with acetone as eluent and without coeluting any "fine".

Choice of the Internal Standard. Several internal standards have been used for the quantitative analysis of oxysterols. To avoid any potential interference with naturally occuring cholesterol oxides but to ensure the same behavior during extraction and LC, we chose to use a homologous sitosterol oxide.

Peak Assignment. In general, analysis by GC-FID alone should only be used as a screening method searching for samples with higher concentrations of oxysterols or to analyze samples with known low matrix background. This can be of use, for example, in quality control to allow for rapid screening of multiple samples both in industry or governmental control laboratories. However, for the quantification of low concentrations of oxysterols or for the analysis of samples with a high matrix background, verification of peak assignment by GC-MS is mandatory.

Conclusions. Considering the biological activities of oxysterols, more information about their occurrence and synthesis in our diet as well as toxicological studies of individual components are needed. These needs require analysis of a larger number of samples, so a fast and reliable screening method is needed to give an estimate of the distribution of oxysterols. The method described here proved to be useful in this respect.

ABBREVIATIONS USED

GC-MS, gas chromatography−mass spectrometry; FID, flame ionization detector; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; MTBE, methyl *tert*-butyl ether; BSTFA, *N,O*-bis(trimethylsilyl)-trifluoroacetamide; TMCS, trimethylchlorosilane, TMSiOH, trimethylsilanol.

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