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TRACE ENRICHMENT AND SEPARATION OF CHOLESTEROL OXIDATION PRODUCTS BY ADSORPTION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method for the liquid chromatographic determination of some oxidation products of cholesterol is described. The oxygenated sterols were concentrated on a short pre-column of Nucleosil NO_2 with hexane as the mobile phase. The separation was performed on a longer column with the same stationary phase but with a hexane–propanol or a hexane–butanol gradient.

Cholest-5-ene-3β,20α-diol, cholest-5-ene-3β,25-diol cholest-5-ene-3β,7α-diol, cholest-5-ene-3β,7β-diol, 3β-hydroxycholest-5-ene-7-one and cholest-5-en-3β-ol were detected at levels as low as 10^{-8} mole $\cdot l^{-1}$. The method was also used for the determination of oxidation products of cholesterol in butter and in heated butter.

INTRODUCTION

Cholesterol is generally recognized as a possible risk factor in the occurrence and progression of coronary heart disease and atherosclerosis. Cholesterol is easily oxidized in the presence of air, especially when heated[1,2]. Some of the oxidized sterols may accumulate in the walls of arteries[3–6], and animal feeding experiments indicate a connection between dietary intake of cholesterol oxidation products and atherosclerosis[7–9]. It is also known that a substantial amount of cholesterol is obtained from the diet and that much food is exposed to heat before consumption. Despite great interest in cholesterol itself, information on its oxidation products and their occurrence, *e.g.*, in foods, is limited. This may be due to the lack of adequate purification and concentration methods. Extraction followed by gas chromatography[3] produced a number of peaks suspected to be oxidation products; however, some of them were found to be artifacts[4] due to the exposure of the samples to air and high temperature. Efficient high-performance liquid chromatography (HPLC) separations of mixtures of oxygenated sterols have been described[10–12] and HPLC separation in combination with enzymatic detection has been suggested[12]. The latter method gives improved detection of sterols with low UV absorption, but its application is restricted to aqueous or alcoholic samples. In the common case of low concentrations in lipid matrices it seems necessary to include a pre-concentration and preferably an enrichment pro-

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cedure. In an unpublished method[13] the triglycerides were removed by freezing and with this procedure the oxidation products could easily be lost by coprecipitation.

This paper describes a method for the determination of oxygenated sterols in foods. It employs a concentration column connected on-line with a separation column. A preliminary study of the formation of some oxygenated sterols in heated butter was made.

EXPERIMENTAL

A sterol mixture containing cholest-5-ene-3β,20α-diol (20α-hydroxycholesterol) ($3 \cdot 10^{-4}$ *M*), cholest-5-ene-3β,25-diol (25-hydroxycholesterol) ($3 \cdot 10^{-4}$ *M*), cholest-5-ene-3β,4β-diol (4β-hydroxycholesterol) ($3 \cdot 10^{-4}$ *M*), cholest-5-ene-3β,7α-diol (7α-hydroxycholesterol) ($4 \cdot 10^{-4}$ *M*), cholest-5-ene-3β,7β-diol (7β-hydroxycholesterol) ($3 \cdot 10^{-4}$ *M*), 3β-hydroxycholest-5-ene-7one (7-ketocholesterol) ($4 \cdot 10^{-4}$ *M*) and cholest-5-ene-3β-ol (cholesterol) ($3 \cdot 10^{-4}$ *M*) was prepared by dissolving the reference substances in hexane–propanol (99.9:0.1) and further diluting with hexane. This mixture will be referred to as "sterol mixture A".

The hydroxycholesterols and 7-ketocholesterol were purchased from Steraloids (Pawling, NJ, U.S.A.). Cholesterol and triolein (glyceryl trioleate) were obtained from Sigma (St. Louis, MO, U.S.A.).

A gradient liquid chromatograph (Spectra Physics SP 8000) was used in all experiments. Spectrophotometric detection was carried out with two UV detectors, an LKB 2138 Uvicord S at 206 nm and with an LDC Spectromonitor III at 210 nm. The detector sensitivities were set to 0.2 a.u.f.s.

The concentration and separation columns were made of stainless steel, with dimensions 40 × 3.9 mm I.D. and 200 × 2.9 mm I.D., respectively. Both columns were packed with 5 μm Nucleosil NO_2 bonded phase material.

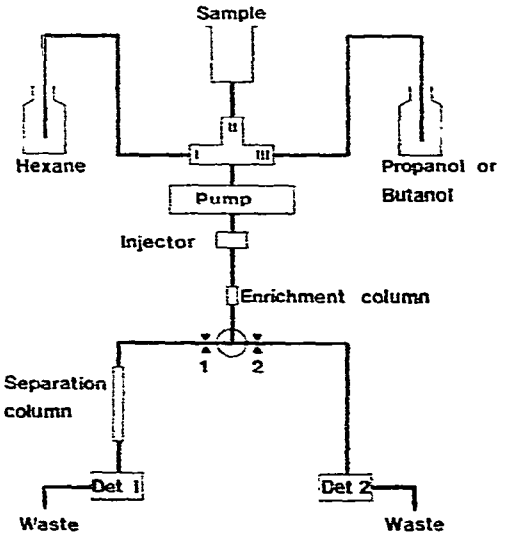

Fig. 1. Chromatographic set-up. 1, 2 = Needle valves; I, II, III = microprocessor-controlled gradient mixing valves.

The experimental set-up, shown in Fig. 1, was used in three modes of operation, each involving changes in mobile phase composition.

Concentration step

The sample was introduced through one of the three mobile-phase inlets of the SP 8000 microprocessor-controlled gradient mixing valves. By appropriate programming, sample volumes of 1.0–1000 ml were injected. Sample volumes of less than 1.0 ml were injected by the valve injector with a syringe. The injection medium was hexane. The outlet from the concentrating column could be directed either to detector 2 or to the separation column by means of a T-connection and two needle valves. During the concentration step valve 1 (to the separation column) was closed and valve 2 was open. Detector 2 (210 nm) gave useful information about components in the sample which were not retarded by the concentration column and about the time needed for a certain sample volume to pass through.

Separation step

Needle valve 1 was opened and needle valve 2 was closed when the signal from detector 2 returned to the baseline. A linear gradient programme from 0 to 5% propanol in hexane or from 0 to 10% butanol in hexane for 30 min at 2.5 ml/min was then activated. Cholesterol and the oxygenated sterols were gradually eluted with increasing proportions of alcohol and were detected at 206 nm.

Re-equilibration

Before the next injection, the system was re-equilibrated with hexane at 2.5 ml/min. Stripping the column with alcohol was completed after about 40 min.

RESULTS

The concentration procedure was studied with a model system which consisted of the diluted sterol mixture A. The chromatograms were evaluated by peak-height measurements. In order to facilitate comparison of peak heights, the results were normalized to the peak height of 7-ketocholesterol.

Pumping speed

Sterol mixture A was diluted to $3 \cdot 10^{-7}$–$4 \cdot 10^{-7}$ *M* and 40.0 ml of the diluted mixture were injected at different pumping speeds. Separations were carried out at 40°C with a linear gradient from 0 to 5% propanol in hexane for 30.0 min at a flow-rate of 2.5 ml/min.

The results in Table I indicate that the enrichment of the sterols is independent of the pumping speed up to at least 7 ml/min. The apparent decrease at 10 ml/min may be due to an error in the volume injected caused by failure of the pumping programme to cope with this high speed. The peak-height variation at pumping speeds below 7.0 ml/min is only slightly higher than that for repeated injections of the same sample volume at constant pumping speed.

Temperature effect

The effect of temperature on the concentration and separation was also ex-

TABLE I

INFLUENCE OF PUMPING SPEED ON CONCENTRATION EFFICIENCY

Pumping speed (ml/min)	*Peak height relative to 7-ketocholesterol*							*Absolute peak height (mm) for 7-keto-chol*
	Chol	*20α-OH-chol*	*25-OH-chol*	*4β-OH-chol*	*7α-OH-chol*	*7β-OH-chol*	*7-keto-chol*	
2.0	0.59	0.60	0.70	0.15	0.86	0.80	1.00	66.3
3.0	0.58	0.61	0.62	0.15	0.86	0.81	1.00	66.0
4.0	0.63	0.68	0.64	0.14	0.84	0.81	1.00	65.5
5.0	0.53	0.64	0.67	0.12	0.87	0.84	1.00	64.5
6.0	0.48	0.60	0.66	0.12	0.90	0.82	1.00	66.8
7.0	0.65	0.67	0.68	0.15	0.85	0.83	1.00	63.0
10.0	0.55	0.59	0.67	0.12	0.79	0.76	1.00	58.0
S (%)*	10	6	4	10	4	3	—	5

* S = Relative standard deviation.

plored. Three temperature settings were used: 75, 40 and 15°C. Separations were carried out as before, but with a constant pumping speed of 1.0 ml/min. A 1.0-ml volume of sterol mixture A diluted to 10^{-5} M was injected.

At 75°C both concentration and separation were affected in a negative way. The peak-height variation for cholesterol, which is the first peak in the chromatogram, was very large and the peaks were also distorted. The recovery, as estimated from peak-height measurements, was 20–30% less than at either 40 or 15°C.

At both 40 and 15°C the concentration was quantitative and the separation was sufficient. The peak-height variation for cholesterol was 10% at 40°C and 7% at 15°C. The peaks were narrower, higher and more symmetrical at the lower temperature. Thus, a temperature of 15°C was chosen for the subsequent experiments.

Concentration effect

One of the most important features of concentration system is its ability to concentrate very dilute solutions with an acceptable recovery. To explore this for the present system the following experiment was carried out.

Five samples, containing the same amount of sterol mixture A (100 μl, 1.0, 10.0, 100.0 and 1000 ml), were prepared. The concentration thus varied from about $3 \cdot 10^{-3}$ to $3 \cdot 10^{-8}$ M. The pumping speed was 5.0 ml/min. Separations were carried out as before. Table II shows that the relative peak heights are independent of dilution, whereas the absolute peak heights show a small decrease due to band broadening in the concentration column. For the most dilute solution several minor "unknown" peaks appeared in the chromatograms. Considering the facile oxidation of cholesterol in dilute solutions[1], it is possible that the peaks may be due to oxidation products other than those contained in sterol mixture A.

Interference from excess of cholesterol

Sterol mixture A was diluted to $3 \cdot 10^{-7}$–$4 \cdot 10^{-7}$ M with hexane in three flasks. Cholesterol was added to total concentrations of $2 \cdot 10^{-6}$, $6 \cdot 10^{-6}$ and $6 \cdot 10^{-5}$ M,

TABLE II

DEPENDENCE OF CONCENTRATION EFFICIENCY ON SAMPLE DILUTION

*Concentration (M)**	*Injection volume (ml)*	*Peak height relative to 7-ketocholesterol*							*Absolute peak height (mm) for 7-keto-chol*
		Chol	*20α-OH-chol*	*25-OH-chol*	*4β-OH-chol*	*7α-OH-chol*	*7β-OH-chol*	*7-keto-chol*	
$a \cdot 10^{-4}$	0.1	0.79	0.62	0.73	0.25	0.82	0.81	1.00	69
$a \cdot 10^{-5}$	1.0	0.93	0.63	0.69	0.24	0.80	0.84	1.00	64
$a \cdot 10^{-6}$	10.0	0.92	0.64	0.69	0.24	0.81	0.78	1.00	61
$a \cdot 10^{-7}$	100.0	0.84	0.71	0.73	0.23	0.82	0.79	1.00	58
$a \cdot 10^{-8}$	1000	0.90	0.79	0.72	0.23	0.86	0.85	1.00	56

* $a = 3$ for cholesterol, 20α-hydroxycholesterol, 25-hydroxycholesterol, 4β-hydroxycholesterol and 7β-hydroxycholesterol; $a = 4$ for 7-ketocholesterol and 7α-hydroxycholesterol.

respectively. Samples of 100 ml were injected. The results, which were obtained under the same conditions as above, are presented in Table III. It can be seen that this excess of cholesterol does not influence the concentration of the other sterols. The chromatogram obtained when cholesterol was present in the largest excess is shown in Fig. 2. The resolution is still very good, and it is not affected by the large cholesterol peak at the beginning of the chromatogram.

Interference from triglycerides

Glycerol triolate represents about 3 % of the lipids in butter and was selected as a model substance for the examination of the concentration selectivity. The most common triglyceride, glycerol tristearate, could not be dissolved in hexane. The gradient mixing valve controlling the flow of propanol (valve III, see Fig. 1) was temporarily connected to a solution of glycerol trioleate in hexane (90.0 mg/ml) during the concentration step. The concentration programme was altered so that the interfering material could be added independently through valve III. Sterol mixture A, diluted to $3 \cdot 10^{-6}$–$4 \cdot 10^{-6}$ *M*, was introduced through valve II. The sample was diluted by the streams through valves I and III. These two valves controlled the ratio of hexane to triolein solution in the enrichment medium. The sample flow-rate was 2.5 ml $\cdot$ min^{-1}, the total flow-rate through the concentration column was 5.0 ml/min and the sample volume was 10.0 ml. The propanol tubing was reconnected and the

TABLE III

DEPENDENCE OF CONCENTRATION EFFICIENCY ON THE AMOUNT OF CHOLESTEROL IN THE ENRICHMENT MEDIUM

Cholesterol (M)	*Peak height relative to 7-ketocholesterol*						*Absolute peak height (mm) for 7-keto-chol*
	20α-OH-chol	*25-OH-chol*	*4β-OH-chol*	*7α-OH-chol*	*7β-OH-chol*	*7-keto-chol*	
$2 \cdot 10^{-6}$	1.00	1.05	0.33	1.00	0.98	1.00	62
$6 \cdot 10^{-6}$	1.05	0.96	0.28	1.02	1.03	1.00	66
$6 \cdot 10^{-5}$	0.95	0.99	0.32	0.99	0.98	1.00	67

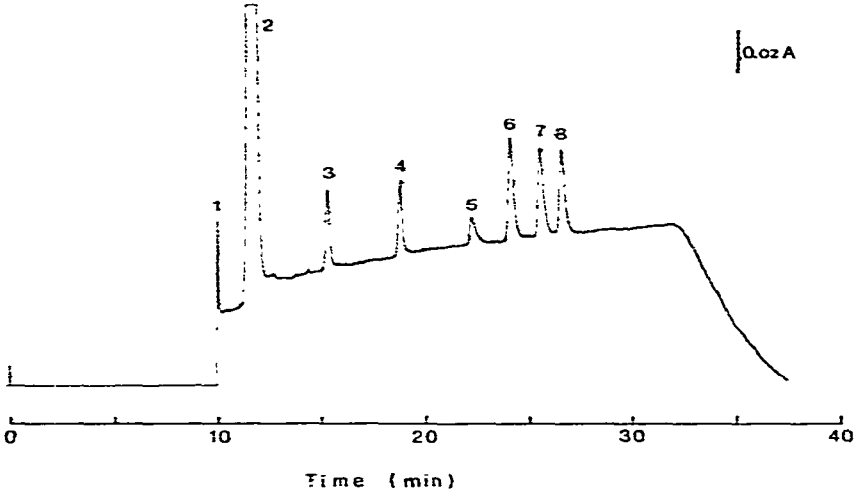

Fig. 2. Concentration and separation of a mixture of oxidized sterols (*ca.* $3 \cdot 10^{-7}$ *M*) with excess of cholesterol ($6 \cdot 10^{-5}$ *M*) in the sample solution. Peaks: 1 = peak due to displacement effect; 2 = cholesterol; 3 = 20α-hydroxycholesterol; 4 = 25-hydroxycholesterol; 5 = 4β-hydroxycholesterol; 6 = 7-ketocholesterol; 7 = 7α-hydroxycholesterol; 8 = 7β-hydroxycholesterol.

original programme was restarted when the concentration had been completed.

A small fraction from the triglyceride solution was also retarded on the concentration column and worsened the separation on the main column. An improvement was achieved when butanol was used instead of propanol and the gradient shape was adjusted. The separation gradient was from 0 to 10% butanol in hexane for 30 min at a flow-rate of 2.5 ml/min. The results from runs with glycerol trioleate levels in the concentration medium of 4.5, 22.5 and 45 mg/ml, respectively, are shown in Table IV.

It was found that the level of triglyceride in the concentration medium affected both the concentration and the separation. The recovery of the early sterol peaks, such as cholesterol and 20α-hydroxycholesterol, decreased with increasing level of triglyceride. The explanation may be that the less polar sterols spread over a larger part of the concentration column owing to a competitive effect of the triglyceride solution. This leads to band broadening and a decreased utilization of the concentration column. The concentration column should therefore have a large over-capacity.

TABLE IV

DEPENDENCE OF CONCENTRATION EFFICIENCY ON THE AMOUNT OF TRIOLEIN IN THE ENRICHMENT MEDIUM

Triolein (mg/ml)	*Peak height relative to 7-ketocholesterol*							*Absolute peak height (mm) for 7-keto-chol*
	Chol	*20α-OH-chol*	*25-OH-chol*	*4β-OH-chol*	*7α-OH-chol*	*7β-OH-chol*	*7-keto-chol*	
0	1.29	1.06	0.91	0.34	0.99	1.02	1.00	72
4.5	0.96	0.67	0.81	0.30	1.00	0.99	1.00	72
22.5	0.85	0.69	0.79	0.29	0.94	0.93	1.00	70
45.0	0.75	0.81	0.78	0.31	0.99	0.95	1.00	70

The separation on the main column was also worsened by components from the trioleate solution, reatained on the concentration column. A broad peak appearing at the front of the chromatogram increased with increasing amount of triglyceride. Prior purification of the triolein solution removes the interference. The competition in the concentration column, on the other hand, seems to be caused by the triglyceride itself. Apart from the additional peak there is, of course, a band broadening when a triglyceride is present during the concentration step, caused by the broader distribution in the concentration column. The separation was acceptable when the level of unpurified trigylceride in the sample solution was below 45 mg/ml.

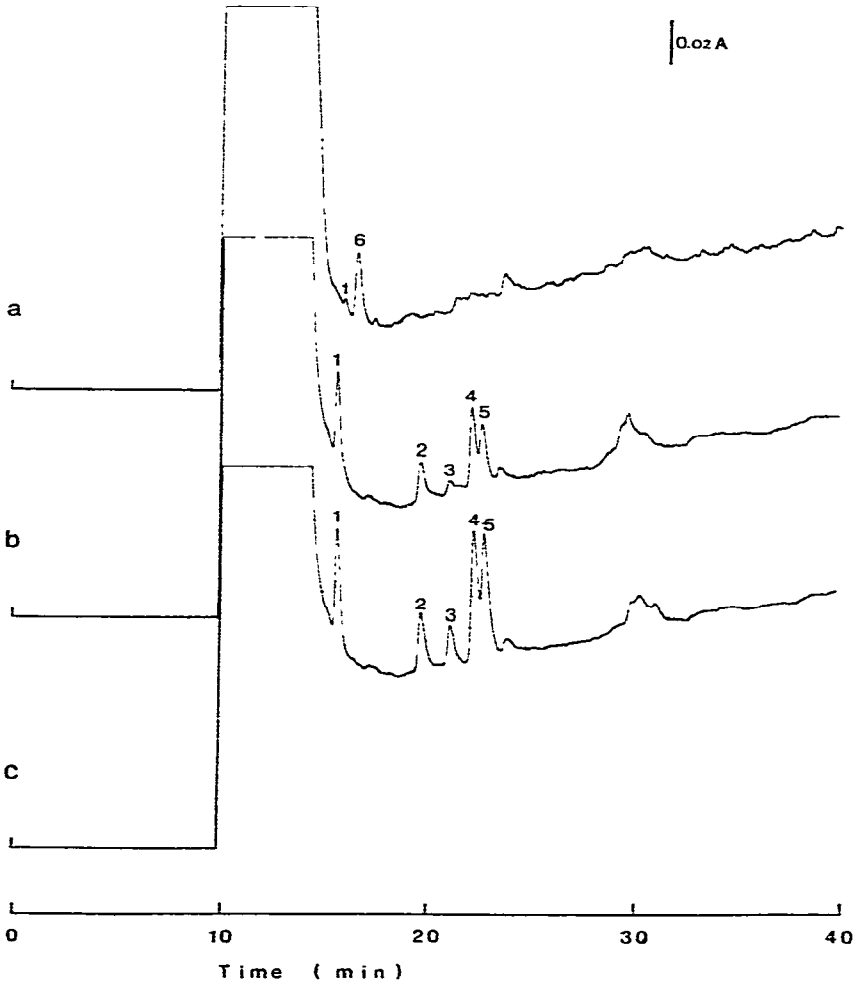

Fig. 3. Concentration and separation of oxidized sterols in heated and unheated butter. (a) Unheated butter; (b) butter heated at 180°C for 5 min; (c) same as b but with standard addition of reference mixture A. Peaks: 1 = 25-hydroxycholesterol; 2 = 4β-hydroxycholesterol; 3 = 7-ketocholesterol; 4 = 7α-hydroxycholesterol; 5 = 7β-hydroxycholesterol; 6 = unknown.

Oxidized cholesterol in butter

The method described above was used for determining oxidized cholesterol in unheated and heated butter. Butter (1.0 g) was heated for 5 min in an open glass vessel kept at 180°C. The butter was dissolved in 100 ml of hexane and the solutions were centrifuged to remove water and filtered to remove particles. Volumes of 25 ml were injected at a pumping speed of 5.0 ml/min. Separations were carried out with a linear gradient from 0 to 10% butanol in hexane for 30 min at a flow-rate of 2.5 ml/min and a temperature of 15°C.

Some of the resulting chromatograms are presented in Fig. 3. Chromatogram a was obtained when a solution of natural butter and b when a solution of heated butter was injected. Chromatogram c was obtained with the same solution as for b, but with addition of reference mixture A.

The separation of the oxidized sterols is still efficient, although the large interfering peak from triglycerides, cholesterol and possibly other compounds in the samples makes UV detection of 20α-hydroxycholesterol impossible. The identity of peak 6 from the unheated butter is not known. Some variation in retention time was sometimes observed. This could be due to a varying degree of displacement of sterols during the concentration step. The amounts of oxidized cholesterol found in heated and unheated butter are given in Table V. The amount of cholesterol in butter is about 2.50 mg/g. Thus about 4% of the cholesterol was oxidized during 5 min at 180°C. The interference from the matrix increased when the heating was prolonged.

DISCUSSION

Trace components can normally be recovered from complex matrices only after lengthy multi-step work-up procedures. Cholesterol and similar lipids in food are usually analysed by chromatographic methods after group separation by different extraction procedures[14–17]. Apart from being laborious these methods are less suitable for the determination of oxidation products as there is a pronounced risk of oxidation during sample preparation. The single-step trace concentration described here is adequate for butter that has been only slightly oxidized. After prolonged heating, there is further degradation, resulting in products that interfere in the method. The nature of these interfering substances is not known, but mass spectrometry has shown that the amount of cholesterol esters with fatty acids increases con-

TABLE V

OXIDIZED CHOLESTEROL CONTENT IN HEATED AND UNHEATED BUTTER

Sample	*Concentration (ug/g)*				
	25-OH-chol	*4β-OH-chol*	*7-keto-chol*	*7α-OH-chol*	*7β-OH-chol*
Unheated	2.6	–	–	–	–
Heated for 5 min at 180°C	34.0	25.6	5.2	19.6	8.4
Heated for 10 min at 180°C	–	5.2	11.2	52.2	19.7

siderably. The selectivity of the concentration step is not sufficient. When some other samples, such as eggs, were analysed, a number of broad peaks appeared in the chromatogram and the determination of trace amounts of oxidized sterols became impossible.

The chromatographic system described is capable of quantitative concentration and separation, even when cholesterol is present in large excess. Triglycerides can be tolerated only in limited concentrations.

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