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Journal of Chromatography A, 736 (1996) 205–210

JOURNAL OF CHROMATOGRAPHY A

Isolation of cholesterol oxidation products from animal fat using aminopropyl solid-phase extraction

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Received 14 March 1995; revised 5 December 1995; accepted 5 December 1995

Abstract

Cholesterol oxidation products were separated from triglycerides and cholesterol in a single step on an aminopropyl solid-phase extraction column. The products were purified by subsequent transesterification and saponification, derivatized to trimethylsilyl ethers and analyzed by gas chromatography. Heated cholesterol-containing fat samples were autoxidized by bubbling air through them. When the flow-rate of air was set at 100 ml/min, the concentration of cholesterol oxidation products in the fat increased to a maximum after 1–2 h and then decreased to almost a zero level after 8 h. The concentration of cholesterol oxidation products in the fat increased over a similar time period, without reaching a maximum, when the flow-rate of air was decreased to 5 ml/min.

Keywords: Cholesterol oxides; Betulin; Triglycerides; Cholesterol

1. Introduction

Many foods contain animal fats, principally of bovine or porcine origin. In prepared foods the fat may be either incorporated during preparation of the food (e.g. in some biscuits and soup mixes) or it is absorbed during cooking (deep frying).

Animal fats are complex mixtures of triglycerides, with lesser amounts of phospholipids and cholesterol (cholest-5-en-3β-ol, see Fig. 1) [1]. Cholesterol, either as a solid, in suspension or in solution, when exposed to air at room temperature is slowly oxidised primarily to a mixture of epimeric 7-hydroperoxides [2]. Autoxidation is more rapid at elevated temperatures. The hydroperoxides readily decompose to 7α- and 7β-hydroxycholesterol and to 7-ketocholesterol. Epoxidation of the Δ^5-double bond in cholesterol yields 5,6α-epoxy-5α- and 5,6β-epoxy-5β-cholestan-3β-ols [2]. Hydration of the epoxycholesterols yields 5α-cholestane-3β,5,6β-triol. Side-chain oxidation may also occur, yielding a variety of 20-, 24-, 25- and 26-hydroperoxides, but this takes place predominantly during autoxidation of crystalline cholesterol [2]. All of these oxidation products are collectively named "cholesterol oxides."

Cholesterol oxidation products have been implicated in the initiation and progression of various

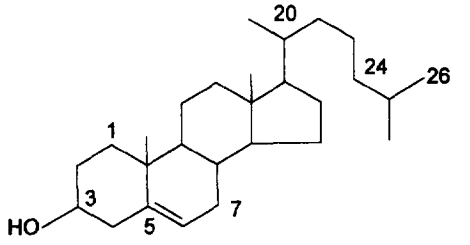

Fig. 1. Structure of cholesterol.

SSDI 0021-9673(95)01322-9

diseases, including atherosclerosis and cancer [3–6]. However, their presence has also been observed in healthy human tissues [7]. They have also been found in fat that had been used for frying [8,9] and in various foods [10–17].

Analysis of cholesterol oxidation products [which are present usually in low concentrations (0.1 to 50 ppm [11])] in fats, usually involves two steps: (a) separation of the sterol fraction from other lipid materials, followed by (b) removal of cholesterol. The initial separation has been accomplished either by saponification–extraction [18,19] or by column chromatography on silica [20]. This was followed by thin-layer [19] or reversed-phase column chromatography [21] to remove cholesterol that would interfere with subsequent gas chromatographic analyses. Despite rigorous clean-up of samples, published gas chromatograms of cholesterol oxidation products obtained from heated fat samples contain large amounts of materials which are probably derived from glyceride [14,22]. Furthermore, autoxidation of cholesterol during analytical procedures, in particular saponification, may result in elevated levels of the oxidation products being reported [23,24].

The aim of the present study was to separate cholesterol oxidation products from cholesterol and triglycerides in a single step. In the method described, cholesterol and triglycerides were rapidly separated from cholesterol oxidation products on an aminopropyl bonded-phase column. The products were purified by transesterification and saponification, derivatized to trimethylsilyl ethers and analyzed by gas chromatography. The method was used to analyse cholesterol oxidation products from heated cholesterol-containing fat through which air was bubbled.

2. Experimental

2.1. Reagents and solvents

Solvents and reagents were of analytical grade. Hexane was a hydrocarbon mixture (Hexanes AR) purchased from Mallinckrodt (Paris, KY, USA) and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Alltech (Deerfield, IL, USA). Cholesterol and oxysterol standards were purchased from Steraloids (Wilton, NH, USA). Betulin (lup-20(29)-ene-3β,28-diol), used as an internal standard, was purchased from Aldrich (Milwaukee, WI, USA). Analytichem Mega Bond-Elut silica (5 g) and Bond-Elut Silica (0.5 g) and Aminopropyl (0.5 g) columns were products of Varian (Harbor City, CA, USA). Cooking fat ("beef dripping") was purchased from a local supermarket.

2.2. Thin-layer chromatography (TLC)

Thin-layer chromatography was performed on aluminium-backed sheets of silica gel 60 (Art. 5553) purchased from E. Merck (Darmstadt, Germany). Solvents used for the separation of glycerides and cholesterol oxidation products were hexane–diethyl ether (9:1, v/v) and hexane–ethyl acetate (3:7, v/v), respectively. Compounds on the plates were revealed by spraying with a 5% solution of phosphomolybdic acid in ethanol. The plates were dried in a stream of warm air and then heated to 200°C on a hot-plate for ca. 0.5 min. The compounds appeared as grey spots on a yellow background.

2.3. Sample preparation

Fat (0.5 g) was dissolved in hexane–dichloromethane (H–DCM) (9:1, v/v; 4 ml) with warming. The solution was applied to an aminopropyl column that had been pre-washed with the same solvent (5 ml). The fat was eluted with: H–DCM (9:1, v/v; 10 ml), fraction 1; H–DCM (1:1, v/v; 6 ml), fraction 2; acetone (5 ml), fraction 3. To maintain a steady flow-rate (ca. 0.5 ml/min), a positive pressure of nitrogen (5–30 cm water) was applied to the head of the column. Fractions 1 and 2 were discarded. Solvent in fraction 3 was removed at 40°C under reduced pressure on a rotary evaporator. The residue was transferred to a capped test tube with H–DCM (1:1, v/v) and the solvent removed under a stream of nitrogen.

Methanolic potassium hydroxide (6%, w/v; 0.5 ml) was added to the test tube and the solution stood at room temperature (20°C) for 30 min. Diethyl ether (1 ml) was then added to the tube (with gentle swirling) followed by distilled water (2 ml) and the contents of the tube gently shaken. The upper layer was removed and the aqueous layer extracted with

diethyl ether (2×0.5 ml). The combined ethereal layers were extracted with 1% aqueous acetic acid (1 ml) and then with distilled water (1 ml). The solvent volume was removed to 1 ml under nitrogen and hexane (0.5 ml) was added and the solution dried with anhydrous sodium sulphate (ca. 0.5 g). Solvent was then removed under a stream of nitrogen.

The residue was taken up in H–DCM (1:1, v/v; 0.5 ml) and applied to a silica (0.5 g) column that had been pre-washed with this solvent (5 ml). The column was eluted with H–DCM (1:1, v/v; 5 ml) (discarded) and then acetone (5 ml). Solvent was removed at 40°C under reduced pressure on a rotary evaporator and the residue transferred to a capped test tube with the minimum amount of DCM that was subsequently removed under a stream of nitrogen.

Methanolic potassium hydroxide (6%, w/v; 0.5 ml) containing 2% (v/v) water was added to the tube, swirled and left for 15 to 24 h at room temperature (20°C). Water (2 ml) was added, the contents gently swirled and then extracted with diethyl ether (3×1 ml). The combined diethyl ether extract was reduced to 1 ml under nitrogen and then hexane (0.5 ml) was added and the solution dried with anhydrous sodium sulphate (0.5 g). Solvent was removed under a stream of nitrogen. The residue was transferred with H–DCM (1:1, v/v) to a 1-ml Reactivial (Labsupply Pierce NZ, Auckland, New Zealand), the internal standard (betulin) added and the solvent removed under a stream of nitrogen.

Trimethylsilyl ether derivatives were prepared by adding 50 μl BSTFA to the Reactivial and heating to 80°C for 1 h [25] in a block heater (Supelco, Bellefonte, PA, USA). On cooling to room temperature, the reaction mixture was diluted with hexane and analyzed by gas chromatography. Cholesterol, 7α- and 7β-hydroxycholesterol, β-epoxycholesterol, 5α-cholestane-3β,5,6β-triol, 7-ketocholesterol and betulin (0.1 mg each) were derivatized and chromatographed in a similar manner.

2.4. Gas chromatography (GC) and mass spectrometry (MS)

GC separations of sterols and their derivatives were carried out with a Hewlett-Packard Model 5980A instrument equipped with a 7673A auto-injector. The column used was a 30 m×0.25 mm DB-1 (0.26 μm) from J&W Scientific (Folsom, CA, USA). Carrier gas was oxygen-free nitrogen, with the column head pressure set at 75 kPa. The injection port (splitless) and detector (flame ionization) were maintained at 200 and 350°C, respectively. The column oven was held at 200°C for 2 min and then programmed to 280°C at 20°C/min, then to 335°C at 5°C/min and held at the maximum temperature for 10 min. GC–MS spectra (70 eV) were recorded on a VG70-250S double focusing magnetic sector mass spectrometer (VG Analytical, Manchester, UK) with an attached Hewlett-Packard 5980A GC system. The above GC conditions were used for these recordings, except that the column inlet pressure was set at 35 kPa.

Retention times of BSTFA-derivatized compounds under the conditions were; 7α-hydroxycholesterol 16.1 min, cholesterol 16.4 min, 7β-hydroxycholesterol 17.3 min, β-epoxycholesterol 17.8 min, 5α-cholestane-3β,5,6β-triol 18.8 min, 7-ketocholesterol 19.4 min and betulin 21.4 min.

To test the repeatability of the method, a solution containing 7α- and 7β-hydroxycholesterol, 5,6-epoxycholesterol and 7-ketocholesterol (15 μg/ml of each compound) in H–DCM (9:1, v/v) was prepared. To fat samples [0.5 g in 4 ml H–DCM (9:1, v/v)] was added 50, 100, 250 or 500 μl of the solution and the mixtures were separated, derivatized and analyzed by GC as described above. To test the recovery of standard compounds 50, 100, 250 or 500 μl of the solution were derivatized and analyzed by GC.

2.5. Autoxidation of heated fat

To remove cholesterol, samples of cooking fat [20 g as a 10% solution in H–DCM (9:1, v/v)] were passed through Mega Bond-Elut columns (that had been conditioned with the above solvent) followed by additional solvent (20 ml). Solvent was removed under reduced pressure from the solution of fat. Cholesterol (0.25%, w/w) was then added to the purified fat and dissolved by heating the mixture until the fat melted. Samples (10 g) of this fat were heated in test tubes held in an oven set at 150°C. When the temperature of the fat reached 150°C, air was bubbled through it for 8 h at either 5 or 100

Table 1
Recovery (%) of cholesterol oxidation products from fat

Added (ppm)		7α-Hydroxy-cholesterol	7β-Hydroxy-cholesterol	5,6-Epoxy-cholesterol	7-Keto-cholesterol
1.5	Mean	87.5	87.9	92.8	87.7
	S.D.	2.3	2.4	2.5	2.6
3.0	Mean	90.2	88.4	93.0	87.9
	S.D.	2.4	2.0	1.8	2.0
7.5	Mean	92.5	92.6	95.0	88.2
	S.D.	1.8	1.8	1.5	2.3
15	Mean	92.4	93.0	94.6	90.1
	S.D.	1.2	2.3	2.0	3.1

ml/min, as measured by an in-line flow meter. After prescribed reaction periods (see Table 1 and Table 2) samples of fat (1 ml) were removed for analysis by the method described above.

3. Results and discussion

3.1. Method development

The separation of cholesterol oxidation products from cholesterol, triglycerides and diglycerides in a single step was achieved by column chromatography, using a short bonded-phase aminopropyl column (containing 0.5 g of solid phase) with H–DCM mixtures as eluent. Most of the triglycerides were removed from the aminopropyl column with H–DCM (9:1) (fraction 1). The remaining triglycerides, diglycerides and cholesterol were removed with H–DCM (1:1), as confirmed by TLC. Cholesterol oxidation products were eluted with acetone (fraction 3). This was confirmed by TLC of this fraction from a spiked sample. The total time required for the H–DCM (9:1, v/v) and the H–DCM (1:1, v/v) to pass through the column was ca. 30 min. Incomplete separation of cholesterol from its oxidation products resulted when DCM was replaced by the appropriate proportions of diethyl ether.

Fraction 3 required further purification as impurities interfered with GC analyses of the cholesterol oxidation products. This interference increased as more samples were analysed and probably arose from the decomposition of polar involatile compounds from the fat in the injection port.

GC and GC–MS analyses showed that fraction 3 contained monoglycerides. These were readily removed by a short, mild alkali-catalysed transesterification followed by column chromatography on silica gel. After removal of the monoglycerides, two major impurity peaks remained. These were shown, by co-injection and MS of the corresponding trimethylsilyl ethers, to be geometric isomers of methyl 9,10-dihydroxystearate [26,27]. These methyl esters were resistant to saponification by overnight treatment with anhydrous methanolic potassium hydroxide, but they were completely hydrolysed if water (2%, v/v)

Table 2
Cholesterol oxidation products (concentration in ppm) in cholesterol-containing fat[a] heated to 150°C through which air was passed at 100 ml/min

Compound[b]	Time (min)										
	0	5	10	15	20	30	40	60	120	240	480
α-Diol	0.0	0.3	1.3	1.7	3.6	7.6	13.4	48.5	90.0	26.2	0.7
β-Diol	0.0	0.4	1.7	2.6	4.4	9.0	16.4	53.7	76.6	24.9	2.0
Epoxy	0.0	0.0	0.6	0.6	1.2	2.5	5.0	6.7	5.9	0.9	0.0
Keto	0.0	1.0	2.1	2.3	3.9	7.5	12.8	38.9	32.4	7.2	0.0

[a] See Section 2.
[b] α-Diol=7α-hydroxycholesterol; β-diol=7β-hydroxycholesterol; epoxy=5,6β-epoxycholesterol; keto=7-ketocholesterol.

was added to the reagent. Samples processed with this modification did not show interferences, mentioned above, during GC analyses. A gas chromatogram of trimethylsilyl ethers of cholesterol oxides from the above-mentioned spiked sample of cooking oil is shown in Fig. 2.

Transesterifications and saponifications were carried out at room temperature to minimise decomposition of the epoxycholesterols and of 7-ketocholesterol [10,22]. No cholesterol oxidation products were detected in the cooking fat or in a freshly extracted sample of beef fat, indicating that cholesterol oxidation did not take place during the analytical procedures.

To test the repeatability of the method, four replicates of samples containing 1.5 to 15 ppm each of 7α- and 7β-hydroxycholesterol, 5,6-epoxycholesterol and 7-ketocholesterol were prepared and analyzed. Regression analysis on the plots of area response ratio versus mass ratio over internal standard yielded correlation coefficient (r^2) values of 0.995, 0.995, 0.993 and 0.984, respectively, for the four compounds. This showed an excellent linear relationship over the concentration range studied.

Mean recoveries of the standard compounds 7α-hydroxycholesterol, 7β-hydroxycholesterol, 5,6-epoxycholesterol and 7-ketocholesterol are given in Table 1. The minimum amount of these compounds that could be detected in the fat was 0.2 ppm under the conditions used in this study.

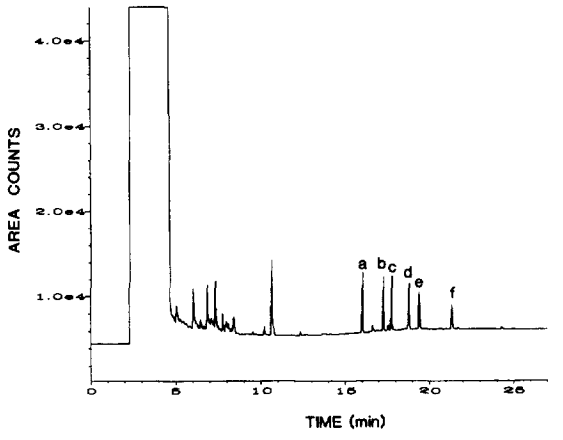

Fig. 2. Gas chromatogram of a mixture of trimethylsilyl ethers of cholesterol oxides and betulin (internal standard). The cholesterol oxides and betulin were dissolved in cooking fat and processed as described in Section 2. a=7α-hydroxycholesterol, b=7β-hydroxycholesterol, c=β-epoxycholesterol, d=5α-cholestane-3β,5,6β-triol, e=7-ketocholesterol and f=betulin.

The method described here can be used for the rapid separation of triglycerides and cholesterol from cholesterol oxidation products in a matrix containing mainly fat. The products required further processing to remove non-volatile impurities that led to interference during GC analyses. Detection of methyl 9,10-dihydroxystearate during the development of the method, even in cooking fat that did not contain cholesterol oxidation products, was unexpected. The diol probably arises from hydrolysis of the corresponding epoxide, formed by hydroperoxide oxidation of oleate. This aspect of lipid oxidation was not studied further here, although it deserves further investigation.

3.2. Cholesterol autoxidation in heated fat

When air was bubbled at 100 ml/min through heated (150°C) cholesterol-containing fat, the principal cholesterol oxidation products were 7-ketocholesterol and the 7-hydroxycholesterols (Table 2). These increased to a maximum over 1–2 h. Lesser amounts of 5,6β-epoxycholesterol were also formed. All of these products had almost vanished at the end of 8 h (Table 2). These conditions were chosen because they approximate those used for estimating fat stability by the active oxygen method [28].

Decreasing the flow of air to 5 ml/min resulted in the formation of cholesterol oxidation products at a slower rate (Table 3). Except for the epoxy compound, their concentration did not reach a maximum during the heating period.

No cholestane-3,5,6-triol, a hydration product of 5,6-epoxycholesterol [29], was detected in this study. This was probably because anhydrous conditions were used.

The rate of formation of oxidation products depends on the extent of contact between the organic compounds and the oxygen. In unstirred conditions, this rate would be limited by the rate of oxygen migration from the atmosphere into the fat [24]. In a previous study [22] the loss of cholesterol was reported to exceed that of the production of oxidation products. Cholesterol not accounted for as identified oxidation products was considered to be lost by

Table 3
Cholesterol oxidation products (concentration in ppm) in cholesterol-containing fat[a] heated to 150°C through which air was passed at 5 ml/min

Compound[a]	Time (min)										
	0	5	10	15	20	30	40	60	120	240	480
α-Diol	0.0	0.3	0.8	1.1	1.4	1.9	3.3	4.8	13.3	24.9	39.0
β-Diol	0.0	0.3	1.3	1.6	2.0	2.6	5.5	8.0	18.6	31.7	44.0
Epoxy	0.0	0.6	1.9	1.5	0.2	0.3	3.1	3.9	7.8	13.0	7.1
Keto	0.0	0.9	1.8	2.1	1.9	2.6	4.8	6.0	15.0	29.6	45.6

[a] See Table 2.

volatilization, degradation to volatile compounds or incorporation into polymers. Similar mechanisms could be used to explain the loss of cholesterol oxidation products in the current studies.

Acknowledgments

The author thanks John Allen for GC–MS analyses.

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