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Gas chromatography–mass spectrometry determination of conjugated linoleic acids and cholesterol oxides and their stability in a model system

T.Y. Yen, B. Stephen Inbaraj, J.T. Chien, B.H. Chen *

Department of Food Science, Fu Jen University, Taipei 242, Taiwan

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ABSTRACT

A gas chromatography–mass spectrometry (GC–MS) method was developed to simultaneously separate cholesterol, eight cholesterol oxidation products (COPs), and two conjugated linoleic acids (9-*cis*,11-*trans*-CLA and 10-*trans*,12-*cis*-CLA) and to evaluate their stability in a model system during heating. Among four capillary columns tested, an Equity-5 column with low-polar stationary phase provided better resolution within 30 min. A high-performance liquid chromatography method was also developed to determine cholesterol hydroperoxides by using a YMC C30 column with diphenyl-1-pyrenylphosphine as fluorescence reagent. No formation of COPs or degradation of cholesterol and CLAs occurred at 100 °C, but the levels of COPs rose drastically at 150 °C. The first-order rate of cholesterol degradation declined following a rise in CLA concentration. For 0-, 100-, and 500-μg/ml CLA levels, the formation profiles of 7-hydroxycholesterol, 7-ketocholesterol, and 5,6-epoxycholesterol at 150 °C were fitted as multiple first-order curves, whereas a single first-order model could adequately describe 7-hydroperoxycholesterol and cholestane-3β,5α,6β-triol formation. A CLA-to-cholesterol mole ratio of 0.49 was required to prevent cholesterol oxidation at 150 °C.

Cholesterol is an important biological compound responsible for synthesis of sexual hormone, vitamin D, and bile acid as well as maintenance of cell membrane permeability. However, cholesterol can form cholesterol oxidation products (COPs)[1] when exposed to light, oxygen, and heat [1,2]. Theoretically, the higher the level of cholesterol, the larger the amount of COPs formed in food products. More than 80 COPs have been reported in nature, yet the most common COPs in food products include 7-ketocholesterol (7-keto), 7α-hydroxycholesterol (7α-OH), 7β-hydroxycholesterol (7β-OH), 5,6α-epoxycholesterol (5,6α-epoxide), 5,6β-epoxycholesterol (5,6β-epoxide), 25-hydroxycholesterol (25-OH), 20-hydroxycholesterol (20-OH), and cholestane-3β,5α,6β-triol (triol) [1,2]. Numerous studies have revealed the intake of COPs in excess can be detrimental to human health and may cause coronary heart disease, cytotoxicity, mutagenicity, and carcinogenicity [1–3]. Therefore, it is imperative to develop suitable analytical methods to determine the variety and amount of COPs in cholesterol-rich food products.

The separation of COPs for analytical purposes has been previously achieved by gas chromatography (GC) or high-performance liquid chromatography (HPLC) [2,4–7]. The former could provide better resolution but requires a time-consuming derivatization procedure to prevent peak tailing and enhance the thermal stability of COPs. Nonetheless, most published reports still choose the GC technique for COPs analysis owing to its good separation ability and higher sensitivity [2,4,5]. Owing to the structural similarity and thermolability of some COPs (hydroxycholesterols), an appropriate choice of capillary columns with suitable operating conditions is critical to attain good resolution using GC.

Incorporation of antioxidants such as butylated hydroxyanisole (BHA) and tocopherol was reported to substantially reduce the formation of COPs in cholesterol-rich food products during heating [2]. Conjugated linoleic acid (CLA), a group of polyunsaturated fatty acids containing 18 C and conjugated double bonds, has received considerable attention during the past decade as several vital biological activities, such as anticancer and enhancement of the

* Corresponding author. Fax: +886 2 29021215.
E-mail address: 002622@mail.fju.edu.tw (B.H. Chen).

[1] *Abbreviations used:* COP, cholesterol oxidation product; 7-keto, 7-ketocholesterol; 7α-OH, 7α-hydroxycholesterol; 7β-OH, 7β-hydroxycholesterol; 5,6α-epoxide, 5,6α-epoxycholesterol; 5,6β-epoxide, 5,6β-epoxycholesterol; 25-OH, 25-hydroxycholesterol; 20-OH, 20-hydroxycholesterol; triol, cholestane-3β,5α,6β-triol; GC, gas chromatography; HPLC, high-performance liquid chromatography; BHA, butylated hydroxyanisole; CLA, conjugated linoleic acid; CT–CLA, 9-*cis*-11-*trans*-octadecadienoic acid methyl ester; TC–CLA, 10-*trans*,12-*cis*-octadecadienoic acid methyl ester; 25-NBD-cholesterol, 25-{*N*-[(7-nitro-2-1,3-benzoxadiazol-4-*yl*)-methyl]amino}-27-norcholesterol; BSA, *N*,*O*-bis(trimethylsilyl acetamide); TMCS, trimethylchlorosilane; TMSI, *N*-trimethylsilylimidazole; DPPP, diphenyl-1-pyrenylphosphine; MS, mass spectrometry; ESI, electrospray ionization; APCI, atmospheric pressure chemical ionization; FID, flame ionization detector; 7α-OOH, 7α-hydroperoxycholesterol; 7β-OOH, 7β-hydroperoxycholesterol; FL, fluorescence; EI, electron ionization; SIM, selective ion monitoring; LC, liquid chromatography; DL, detection limit; S/N, signal-to-noise ratio; QL, quantitation limit; ANOVA, analysis of variance; CV, coefficient of variation.

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immune system as well as prevention of atherosclerosis, have been reported [8]. Bauman and coworkers [9] reported that the production of butter with enhanced CLA could inhibit breast cancer formation in an animal model. In addition, CLA has been shown to reduce arachidonic acid formation and retard cyclooxygenase activity so that the cancer risk could be lowered through reduction of inflammation precursors such as prostaglandin, thromboxane, and leukotriene [10]. Therefore, the potential of using CLA as an antioxidant to prevent chronic disease cannot be ignored.

Most CLAs are naturally present in meat and dairy products, especially for ruminant products in which 9-*cis*-11-*trans*-octadecadienoic acid is the most abundant and constitutes 75 to 90% of total CLAs [11]. Of the various meat products, mutton contained the highest level of CLA (4.32–19.0 mg/g lipid), followed by beef (1.2–10.0 mg/g lipid) [12]. However, only a slight amount of CLA (0.6–0.9 mg/g lipid) was present in vegetable oil and butter [13]. Luna and coworkers [14] compared stability of linoleic acid and CLA in a dark room at 30 °C and found that the oxidation stability of linoleic acid could be enhanced in the presence of CLA. Thus, the incorporation of CLA into an oil system to enhance its oxidation stability should be explored, and to determine the effect of CLA on cholesterol oxidation, a simultaneous separation of cholesterol, CLAs, and COPs is essential. Therefore, the objectives of this study were (i) to develop a GC method for simultaneous separation of cholesterol, two CLAs, and eight COPs by comparing four different capillary columns and (ii) to evaluate the stability of cholesterol in the presence of CLA in a model system during heating.

Materials and methods

Materials

Fatty acid and CLA standards, including linoleic acid methyl ester, 9-*cis*-11-*trans*-octadecadienoic acid methyl ester (CT–CLA), and 10-*trans*,12-*cis*-octadecadienoic acid methyl ester (TC–CLA) with a high purity (≥99%), were procured from Nu-Check Prep (Elysian, MN, USA). Cholesterol and COP standards, including 5,6α-epoxide, 5,6β-epoxide, 7-keto, triol, 25-OH, 20-OH, and internal standard 5α-cholestane, were obtained from Sigma (St. Louis, MO, USA). Both 7α-OH and 7β-OH were obtained from Steraloids (Wilton, NH, USA). The external standard 25-{*N*-[(7-nitro-2-1,3-benzoxadiazol-4-*yl*)-methyl]amino}-27-norcholesterol (25-NBD-cholesterol) was purchased from Avanti Polar Lipid (Alabaster, AL, USA). The purities of all these standards were from 95 to 99%. The derivatizing agent Sylon BTZ containing *N*,*O*-bis(trimethylsilyl acetamide) (BSA), trimethylchlorosilane (TMCS), and *N*-trimethylsilylimidazole (TMSI) at a 3:2:3 ratio was supplied by Supelco (Bellefonte, PA, USA). The fluorescence reagent diphenyl-1-pyrenylphosphine (DPPP) was obtained from Tong-Ren Research Institute (Tokyo, Japan).

The HPLC-grade solvents, including methanol, diethyl ether, hexane, acetone, ethyl acetate, and *n*-butanol, were obtained from Lab-Scan (Gliwice, Poland). Pyridine was obtained from J.T. Baker (Phillipsburg, NJ, USA). Deionized water was made using a Milli-Q water purification system (Millipore, Bedford, MA, USA). BHA was purchased from Nacalai Tesque (Kyoto, Japan). Paraffin oil was obtained from Riedel-de-Haën (Seelze, Germany).

Instrumentation

The GC system (model 6890) equipped with a mass spectrometer (model 5973) was obtained from Agilent Technologies (Palo Alto, CA, USA). The HPLC–mass spectrometry (MS) system (Agilent 1100 series) is composed of a G1316A column temperature controller, a G1379A degasser, a G1311A quaternary pump, a G1312A binary pump, a G1315B photodiode array detector, and a 6130 quadrupole mass spectrometer with multimode ion source (electrospray ionization [ESI] and atmospheric pressure chemical ionization [APCI]). The on-line degasser (DP-4010) was obtained from Sanwa-Tsusho (Tokyo, Japan). The injector (model 7161) was obtained from Rheodyne (Rohnert Park, CA, USA). The PU-980 and PU-1580 pumps, as well as the 821-EP model fluorescence detector, were purchased from Jasco (Tokyo, Japan). The oil bath heater (H-271) was obtained from Toyo Seisakusho Kaisha (Chiba, Japan). The rotary vacuum evaporator (N-1) was obtained from Eyela (Tokyo, Japan). The sonicator (DC 400) was supplied by Chuan-Hua (Taipei, Taiwan). The solid phase C18 cartridge (Strata C18-E, 500 mg/3 ml, 55 μm, 70 Å) was obtained from Phenomenex (Torrance, CA, USA). The silica cartridge (SampliQ silica, 500 mg/6 ml, 45 μm, 60 Å) was obtained from Agilent Technologies.

Simultaneous separation of cholesterol, CLAs, and COPs by GC

Initially, four different capillary columns—Agilent DB-1 nonpolar column (60 m × 0.32 mm i.d., 0.25 μm film thickness, 100% dimethylpolysiloxane), Agilent HP-5MS low-polar column (30 m × 0.25 mm i.d., 0.2 μm film thickness, 5% diphenylpolysiloxane/95% dimethylpolysiloxane), Supelco MDN-5 low-polar capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness, 5% diphenylpolysiloxane/95% dimethylpolysiloxane), and Supelco Equity-5 low-polar column (30 m × 0.25 mm i.d., 0.25 μm film thickness, 5% diphenylpolysiloxane/95% dimethylpolysiloxane, low bleed)—were compared for separation efficiency of cholesterol, two CLAs, and eight COPs. Two temperature-programming methods based on Lee and coworkers [15] and Liu and coworkers [16] were modified and used for separation. The former used an Agilent HP-5MS column to separate seven COPs within 16 min with injector temperature 280 °C, split ratio 5:1, GC–MS interface temperature 270 °C, and column temperature at 220 °C in the beginning, increased to 275 °C at 15 °C/min and to 280 °C at 1 °C/min and maintained for 2 min, and to 290 °C at 5 °C/min and maintained for 10 min. The latter used an Agilent HP-88 column to resolve 17 fatty acid methyl ester standards within 31 min with injector temperature 240 °C, split ratio 10:1, detector (flame ionization detector [FID]) temperature 250 °C, and column temperature at 170 °C initially, maintained for 24 min, raised to 220 °C at 7.5 °C/min and to 230 °C at 10 °C/min and maintained for 5 min. The separation efficiency was evaluated based on retention factor (k) and separation factor (α) values determined by $k = (t_R - t_o)/t_o$ and $\alpha = (t_{R2} - t_o)/(t_{R1} - t_o)$, where t_o is the retention time of the solvent peak, t_R is the retention time of peak 1 or peak 2, t_{R1} is the retention time of peak 1, and t_{R2} is the retention time of peak 2.

Determination of cholesterol hydroperoxides by HPLC

A method based on Sohn and coworkers [17] was modified to separate 7α-hydroperoxycholesterol (7α-OOH) and 7β-hydroperoxycholesterol (7β-OOH) by using a Waters YMC C30 polymeric column (250 × 4.6 mm i.d., 5 μm). A mobile phase of 100% methanol was used with flow rate at 0.8 ml/min, column temperature at 25 °C, and injection volume at 20 μl. A postcolumn reaction device was adopted as well by pumping fluorescence reagent containing a mixture of 100 mg of BHA and 12 mg of DPPP reagent in 400 ml of methanol/*n*-butanol (50:50, v/v) into a reaction coil (1.5 m × 0.25 mm i.d.) at a flow rate of 0.7 ml/min to mix with eluate from the column, followed by immersing the mixture in an 80 °C water bath for reaction, cooling in a coil (0.5 m × 0.25 mm i.d.), and detecting 7α-OOH and 7β-OOH with fluorescence (FL) at the excitation wavelength 352 nm and the emission wavelength 380 nm. The external standard 25-NBD-cholesterol was also detected by