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Simultaneous Analysis of Cholesterol Oxidation Products (COPs) in Powdered Milk Using HPLC/UV-Vis

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Cholesterol and cholesterol oxidation products (COPs) may accumulate in foods of animal origin during processing or storage. An effective and sensitive analytical method was developed by increasing the UV absorption of compounds through derivatization by attaching a chromophore to the functional groups of cholesterols (cholesterol, 20-hydroxycholesterol, 7-ketocholesterol, cholestane-3β-5α-6β-triol, 25-hydroxycholesterol, and 5,6α-epoxycholesterol). The influences of the reaction time, volume of reaction solvent, amounts of derivatizing reagent, and extraction solvents were investigated, as they may influence the reaction and extraction yield. The derivatized COPs were analyzed simultaneously on a C18 column (2.1 mm i.d. × 100 mm length, 3.5 μm particle size) using a gradient elution with water and acetonitrile. The derivatized COPs showed increased sensitivity and selectivity in HPLC/UV-Vis. The LOD and LOQ were in the concentration ranges of 0.018-0.55 mg/kg and 0.059-1.84 mg/kg from the powdered milk. And the accuracy and precision were 78.1-116.7% and 1.1-9.9%, respectively.

Key Words : Cholesterol, Cholesterol Oxidation Products (COPs), HPLC/UV, Derivatization/Extraction

Introduction

Cholesterol is a structurally unsaturated alcohol found in most foodstuffs of animal origin. Cholesterol is also distributed in mammalian muscle, brains, nerve tissue, bile, blood, and fat, and is an important component of phospholipids and biological membranes. It also blocks the destruction of red blood cells and plays an important role in the physiological functions of mammals.[1] However, cholesterol can be easily oxidized by exposure to heat, or air and light, or by free radical initiators, as well as several other complex factors.[2,3] Animal-origin foodstuffs, which are processed into various products (*e.g.* whole milk powder, egg powder, freeze-dried meat, and heated animal fats) or stored in the presence of oxygen, heat, and light, are the main sources of cholesterol oxides in the human diet.[4]

More than 80 species of cholesterol oxidation products (COPs) formed by a chain reaction of oxygen radicals or free radicals generated by hydroperoxide of unsaturated fatty acid were reported.[6-13] Cholesterol oxides have been reported to have harmful influences on people, such as cytotoxicity, atherosclerosis, interference with cholesterol metabolism, and mutagenic and carcinogenic activities.[6-9] The cholesterol oxidation products (COPs) that are most frequently generated in foods, are 7-ketocholesterol, 7α-7β-hydroxycholesterol, 5,6α-epoxycholesterol, 5,6β-epoxycholesterol, 25-hydroxycholesterol, 20α-hydroxycholesterol, and cholestane-3β-5α-6β-triol.[10] 7-ketocholesterol inhibits the growth of fibroblasts, which are involved in human cholesterol biosynthesis,[11] while 5,6α-epoxycholesterol and 5,6β-epoxycholesterol induce carcinogenic mutations, and 25-hydroxycholesterol and cholestane-3β-5α-6β-triol cause atherosclerosis and damage the surfaces of the aorta.[12] Therefore, it is necessary to monitor the presence of COPs, which are contained at low concentrations (ng/kg to μg/kg) in foods.

Because COPs have different functional groups that result in diverse polarities and chemical properties in the molecules, and since they have similar chemical, spectrophotometric, and fragmentation characteristics, they are difficult to analyze in complex matrices.[13] Although COPs are analyzed by gas chromatography (GC) or gas chromatography/mass spectrometery (GC/MS), the inlet, column, detector, and interface are run at high temperature, which may produce artifacts due to the thermal degradation of cholesterol and cholesterol oxides.[14] Derivatization allows volatile substances to be analyzed by GC or GC/MS analysis, but requires a relatively complex process.[15-18] In addition, because COPs are relatively nonpolar compounds, the analysis of COPs by LC/ESI-MS (Liquid Chromatography/ Electrospray Ionization-Mass Spectrometry) shows poor sensitivity. Alternatively, although the analysis of COPs by LC/APCI-MS (Liquid Chromatography/Atmospheric Pressure Chemical Ionization-Mass Spectrometry) is relatively effective and does not require derivatization,[19,20] it has disadvantages associated with expensive equipment and intractability compared to HPLC/UV-Vis. But, because COPs have chromophores with little or very weak absorption, HPLC/UV-Vis analysis shows low sensitivity.[21,22] Although a few derivatization methods of cholesterol by attaching benzoylchloride followed by HPLC/UV-Vis analysis have been reported, complex derivatization procedures have been needed.[23,24]

In this paper, the five COPs (20-hydroxycholesterol, 7-ketocholesterol, cholestane-3β-5α-6β-triol, 5,6α-epoxycholesterol, 25-hydroxycholesterol) mainly detected in foods were selected to develop an effective analytical method by

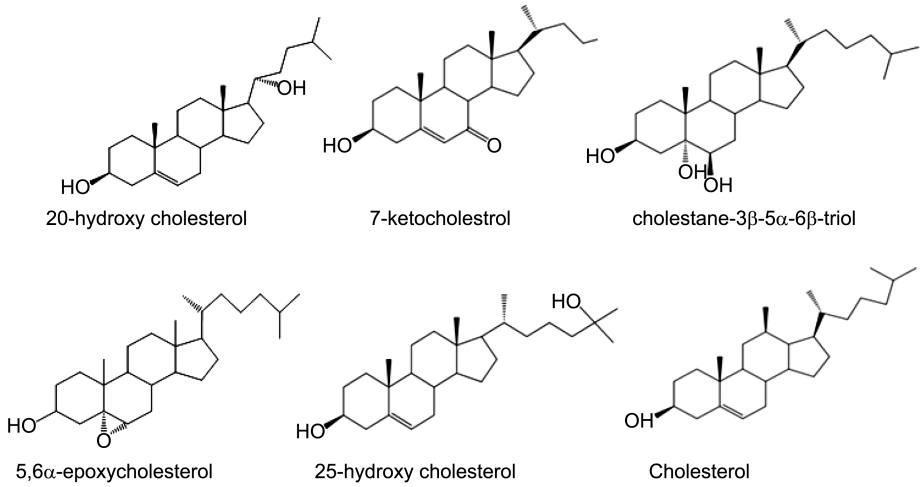

Figure 1. Chemical structures of cholesterol and cholesterol oxidation products (COPs).

HPLC/UV-Vis (Figure 1). A simple method of benzoylchloride derivatization modified for cholesterol and COPs shows increased sensitivity to HPLC/UV-Vis. The developed extraction and derivatization method can be useful to analyze five COPs by HPLC/UV-Vis in powdered milk.

Experimental

Reagents, Materials, and Instruments. Cholesterol and COPs (20-hydroxycholesterol, 7-ketocholesterol, cholestane-3β-5α-6β-triol, 5,6α-epoxycholesterol, 25-hydroxycholesterol) were purchased from Sigma-Aldrich (St Louis, MO, USA). HPLC-grade organic solvents, such as methanol, acetonitrile, ethyl acetate, and chloroform, were obtained from J. T. Baker (Phillipsburg, NJ, USA). High-purity pyridine and benzoylchloride were obtained from Sigma-Aldrich (St Louis, MO, USA). Hydrochloric acid was obtained from DaeJung Chemicals & Metals (Gyeonggi-do, Korea). The working standard solutions were prepared by dissolving cholesterol and COPs in chloroform (1000 μg/mL), and stored in a refrigerator (−20 °C). Before use, these standard solutions were diluted with chloroform.

Polypropylene conical tubes were obtained from Falcon (Austin, Texas, USA). An MMS-3010 centrifuge was obtained from Han-Il Scientific Co. Ltd. (Seoul, Korea), and centrifuge tubes (50 mL) were obtained from Nalgene (New York, USA). GF/B glass filter paper was obtained from Whatman (Maidstone, UK), and a vacuum manifold was obtained from Supelco (Bellefonte, PA, USA). A TurboVap LV nitrogen concentrator was obtained from Caliper Lifescience (Seattle, WA, USA), and a vortex mixer was obtained from Vision Scientific (Bucheon, Korea).

An Agilent 1100 series HPLC system (Palo Alto, CA, USA) equipped with a binary pump was connected to an Agilent G1313A autosampler and G1313A UV-Vis detector. The GC/MS analysis was done on an Agilent 6890N gas chromatograph (Palo Alto, CA, USA) equipped with an Agilent 5973N mass selective detector.

Sample Preparation. Sample preparation modified the published method was used.[25] A 5 g amount of milk powder was prepared in polypropylene conical tubes (50 mL). 10 mL of water and 18 mL of 2-propanol were added to the sample tube and mixed for 3 min at 250 rpm. 14 mL of *n*-hexane was added and shaken for 10 min at 250 rpm. Then, centrifugal separation was performed for 15 min at 3500 rpm, and the upper layer was transferred to a new tube. The aqueous layer was re-extracted with 14 mL of *n*-hexane.

The collected supernatant was mixed with 9 mL of 0.5 M Na_2SO_4 in water. The supernatant obtained by the centrifugal separation for 5 min at 3500 rpm was collected in a round-bottom flask of 100 mL and evaporated using a rotary evaporator. Redissolving solution with 1 mL of *n*-hexane (two times) was added to 600 μL of 2 M methanolic KOH for transesterification reaction, and then shaken for 1 min using a vortex mixer. The system was left to stand at room temperature for 30 min for reaction, and 600 μL of water was added. After 30 min, when the layer separation was complete, the supernatant was vaporized using a nitrogen evaporator at 40 °C, and then 500 μL of chloroform was added.

An amine (6 cc, 500 mg) cartridge (Waters, Milford, MA, USA) was connected to a vacuum manifold and conditioned by a solution containing 5 mL of *n*-hexane, and then 5 mL of the sample was loaded into the cartridge. The sample was washed with 20 mL of *n*-hexane, 5 mL of *n*-hexane:MTBE (5:1), and 5 mL of *n*-hexane:MTBE (3:1), consecutively. The sample was eluted with 10 mL of *n*-hexane: ethyl acetate (1:4) and 10 mL of acetone. The eluent was completely vaporized by a nitrogen evaporator at 40 °C.

Derivatization Reaction and Solvent Extraction. Prior to HPLC/UV-Vis analysis, standards of cholesterol and COPs in chloroform were spiked in a glass test tube to obtain concentrations of the analyte on the μg/mL scale, after which the solvent was completely dried, and 50 μL of pyridine and 10 μL of benzoylchloride were added (Figure 2). Afterward, the sample was mixed by a Vortex mixer for 2 min, and incubated using a heating block for 80 min at 80 °C. After the reaction vessel was cooled to room temperature,

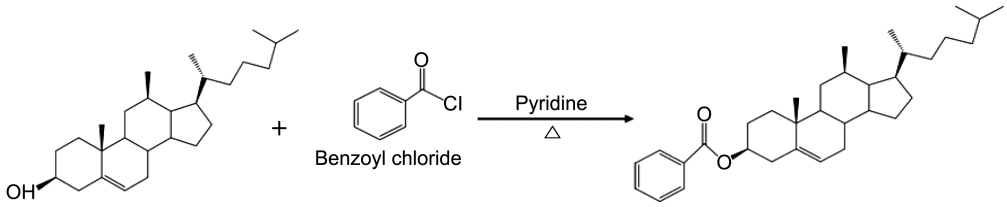

Figure 2. Derivatization scheme of cholesterol.

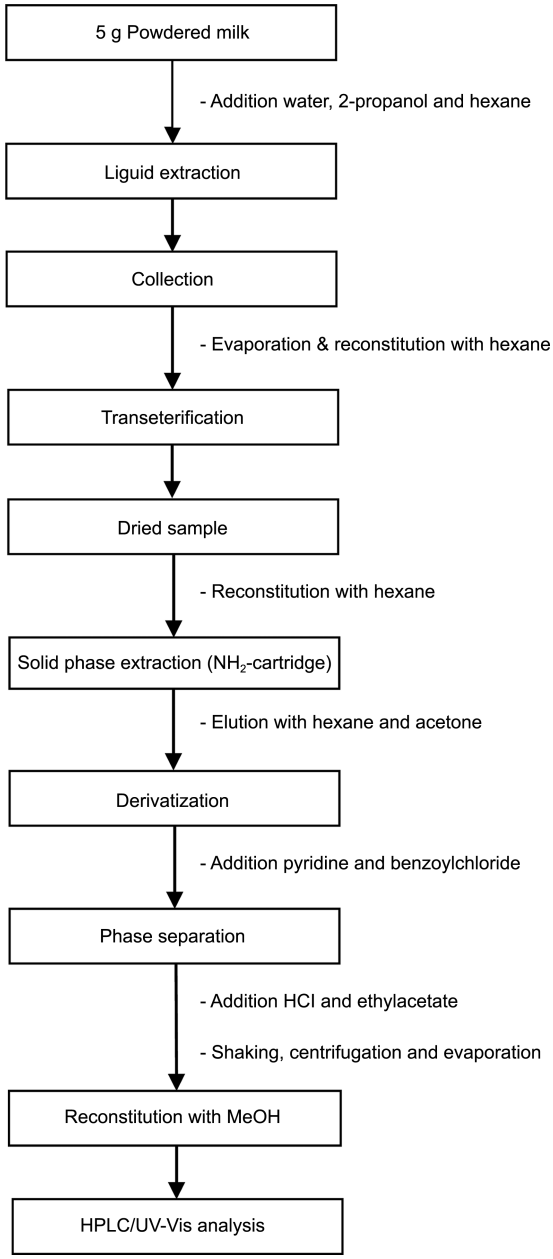

Figure 3. Derivatization/extraction procedure of cholesterol and COPs.

5 mL of ethyl acetate and 5 mL of 0.1 M HCl were added, and the tube was shaken for 5 min at 200 rpm.

Then, centrifugal separation was performed for 5 min at 3500 rpm, and the upper layer was collected in a new tube. The solvent was completely vaporized by using a nitrogen evaporator at 40 °C. The residue was dissolved with 100 μL of MeOH and then filtrated through a 0.45 μm disk filter for LC/UV-Vis analysis (Figure 3).

Conditions of Instrumental Analysis. A reverse-phase Eclipse Plus C18 was used as the analytical column (2.1 mm i.d. × 100 mm length, 3.5 μm particle size). Mobile phases A (water) and B (acetonitrile) were used with a flow rate of 0.3 mL/min with a gradient system. The separation was achieved with the following linear gradient system: at 0 min A:B = 5:95 (v/v), 4.1 min A:B = 0:100, 44 min A:B = 5:95, followed by post time (16 min) to allow for re-equilibration of the column. The injection volume was 10 μL, and the measured wavelength was set at 230 nm.

GC/MS analysis was performed for the structural identification of the derivatized COPs. GC analysis was performed on an HP-5MS capillary column (30 m length × 0.25 mm i.d., 0.25 μm film thickness) with helium as a carrier gas (1.2 mL/min). The oven temperature was set to 60 °C for 1 min, and increased at a rate of 10 °C/min to 290 °C, and increased again at a rate of 0.5 °C/min to 300 °C, where it was maintained for 5 min. The injection volume and temperature were 2 μL and 280 °C, respectively. The temperatures of the transfer line, ion source, and quadrupole analyzer were maintained at 280, 230, and 150 °C, respectively. In the full-scan mode, electron ionization (EI) mass spectra in the range of *m/z* 40-800 were recorded at an electron energy of 70 eV.

Calibration Curves. For establishing the calibration curves, the samples were spiked with standard solutions of the six concentration levels with the concentration range of 0.059-56.0 mg/kg in blank powdered milk sample for five cholesterol oxidation products and then the analytes were extracted and derivatized by the established method. The calibration curves were made from the peak area of the derivatized COPs in HPLC.

The calibration curves was made by the peak area which is subtracted the one of the blank sample from the peak area of the standard spiked sample, when the analytes were being in the blank powdered sample.

Results and Discussion

HPLC/UV-Vis Analysis. Before derivatization, the maximum UV absorbance of cholesterol and COPs was approximately 206 nm. Because 206 nm of the wavelength is the cut-off wavelength of the mobile phase typically used in a reversed-phase chromatographic system for HPLC/UV-Vis analysis, it was not desirable to determine with good sensitivity and efficiency. Therefore, in order to compensate

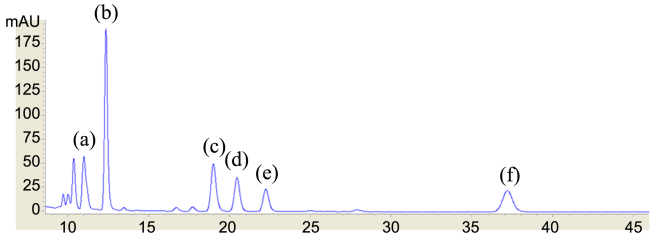

Figure 4. HPLC/UV-Vis chromatogram of derivatized cholesterol and COPs: (a) 20α-hydroxycholesterol, (b) 7-ketocholesterol, (c) cholestane-3β-5α-6β-triol, (d) 5,6α-epoxycholesterol, (e) 25-hydroxycholesterol, and (f) cholesterol.

for these shortcomings, the derivatization of cholesterol and COPs with benzoylchloride was carried out, and the maximum wavelengths of the derivatized substances were shifted to around 230 nm, which does not interfere with the mobile phase.

As shown in Figure 4, the derivatized compounds were effectively separated using a reversed phase HPLC/UV-Vis system, indicating 20-hydroxycholesterol at 10.9 min; 7-keto cholesterol at 12.3 min; cholestane-3β-5α-6β-triol at 18.9 min; 5,6α-epoxycholesterol at 20.3 min, and 25-hydroxy cholesterol at 22.1 min.

Identification of Derivatized COPs. Cholesterol and COPs derivatized with benzoyl chloride were identified through GC/MS. But, because of the limitation of the electron impact (EI) ionization of GC/MS, molecular ions were not detected. Instead, the commonly characterized fragment pattern was observed of the benzoic acid group(s) in the molecular structures of the derivatized substances being removed. For 20α-hydroxycholesterol, [M-2$C_7H_5O_2$-

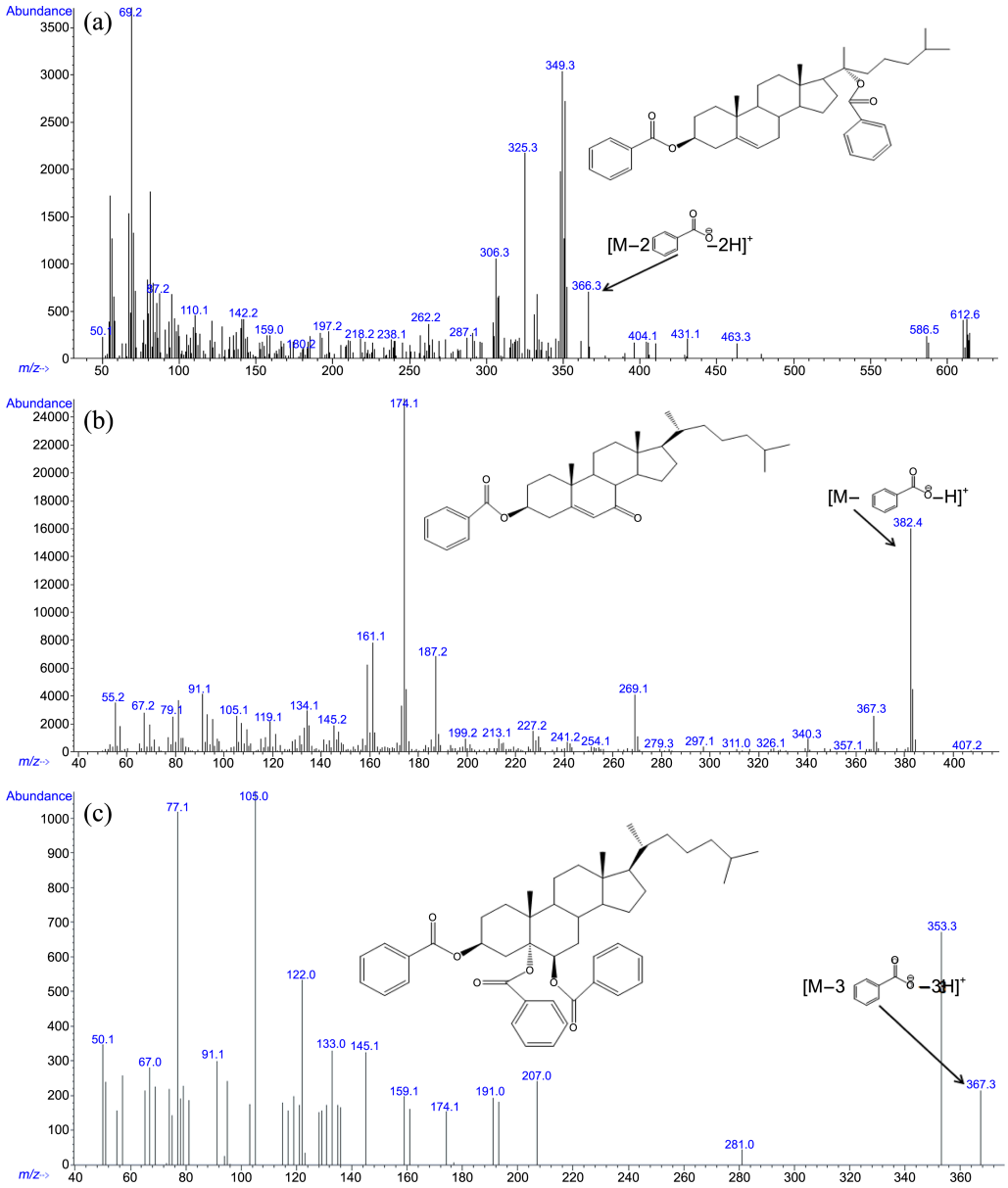

Figure 5. GC/MS mass spectra for derivatized COPs: (a) 20α-hydroxycholesterol, (b) 7-ketocholesterol, (c) cholestane-3β-5α-6β-triol, (d) 5,6α-epoxycholesterol, and (e) 25-hydroxycholesterol.

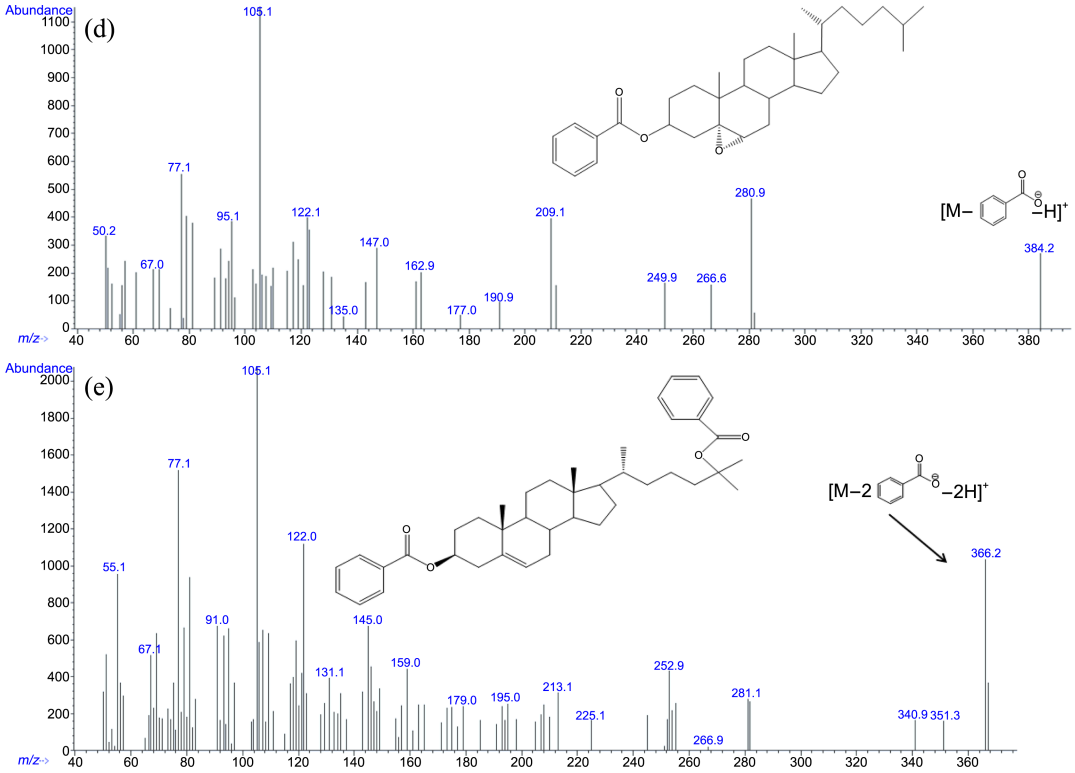

Figure 5. Continued

$2H]^+$ was detected at *m/z* 355.1, as were the following: 7-keto cholesterol: $[M\text{-}C_7H_5O_2\text{-}H]^+$ at *m/z* 382.4; cholestane-3β-5α-6β-triol: $[M\text{-}3C_7H_5O_2\text{-}3H]^+$ at *m/z* 367.3; 5,6α-epoxycholesterol at *m/z* 384.0; 25-hydroxy cholesterol: $[M\text{-}2C_7H_5O_2\text{-}2H]^+$ at *m/z* 366.2 (Figure 5).

Optimization of Experiment Parameters. The reaction time, extraction solvent type, amount of reactant, and amount of derivatization reagent were varied, and the derivatization and extraction experiments were performed to optimize the experimental conditions to give the maximum yield.

Reaction Time. The other parameters were fixed to the conditions indicated in the section describing the derivatization reaction and solvent extraction, and only the reaction time was varied to 20, 30, 40, 60, 80, and 100 min. The relative peak areas of HPLC/UV-Vis according to each reaction time are shown in Figure 6. Although 20-hydroxy cholesterol and 25-hydroxycholesterol showed slower reaction rates, most substances reached the maximum peak area within 80 min.

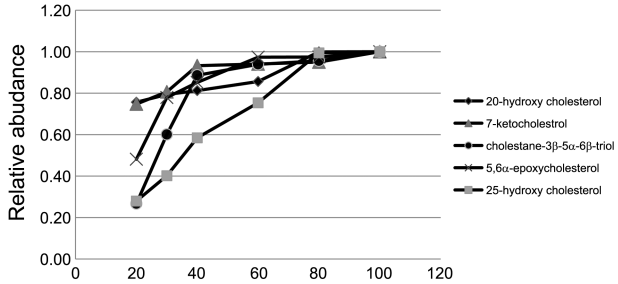

Figure 6. Comparison for derivatized COPs according to reaction time.

Amount of Reaction Solvent. Pyridine, which acts as a reaction solvent and Lewis base, was chosen for the derivatization reaction. The other conditions were fixed to the conditions indicated in the section describing the derivatization reaction and solvent extraction, except that the amounts of pyridine were varied to 50, 100, 200, and 500 μL. The reaction efficiency was evaluated by comparison of the HPLC peak area.

Figure 7 shows the influence of the pyridine amount on the relative peak intensity, with most of the analytes showing the lowest abundances at 500 μL of pyridine. 20-hydroxy cholesterol and 7-ketocholesterol showed higher peak abundance at 200 μL, but cholestane-3β-5α-6β-triol and 25-hydroxy cholesterol showed relatively low abundance. 50 μL and 100 μL of pyridine resulted in even higher peak intensities for the five analytes. In order to remove pyridine effectively, and to thoroughly dissolve the residue of COPs extracted from powdered milk, 100 μL of pyridine was selected as the optimized amount.

Amount of Derivatization Reagent. Benzoylchloride was used as a chromophore in order to increase the sensitivity in HPLC/UV-Vis and to avoid overlap of the wavelength of the UV absorption with the mobile phase solvent of HPLC/UV-Vis. The other conditions were fixed to conditions indicated in the section describing the derivatization reaction and solvent extraction, except that the amount of benzoylchloride used as a derivatization reagent was varied to 2, 5, 10, and 15 μL. The efficiency of the reaction was examined by comparing peak area obtained from HPLC/

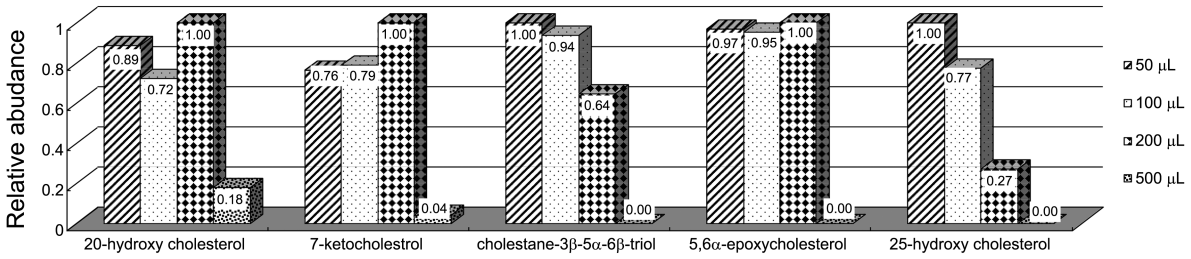

Figure 7. Comparison for derivatized COPs according to volume of pyridine.

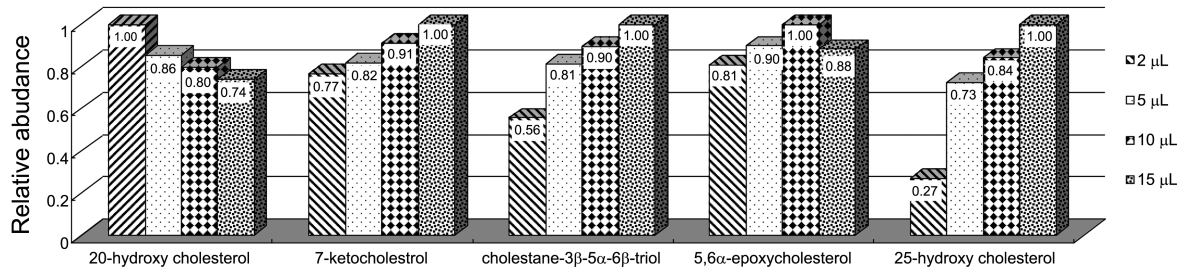

Figure 8. Comparison of derivatized COPs according to volume of benzoylchloride.

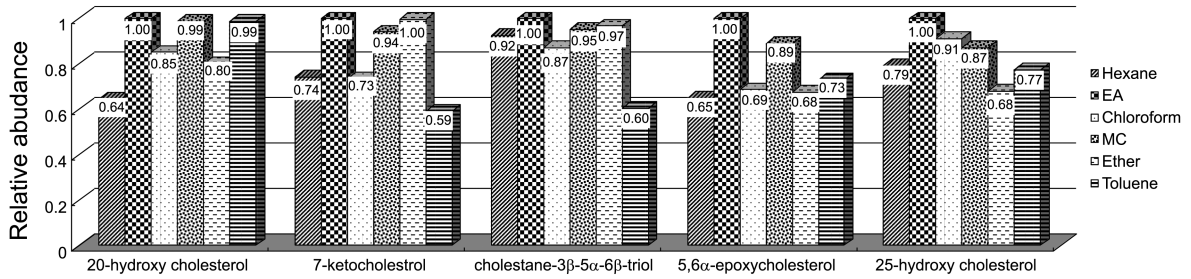

Figure 9. Comparison of relative extraction yield for cholesterol and derivatized COPs according to extraction solvent: Hexane, EA (ethlyacetate), Chloroform, MC (methylene chloride), Ether (diethylether), and Toluene.

UV-Vis (Figure 8). Because the residual matrix rather than the analytes may be consumed, the amount of material needed could not be predicted, an excess of the derivatization material is typically added for the reaction. However, since too much derivatization reagent is not effective for next steps, the amount of reagent must be controlled.

As shown in Figure 8, most of the analytes showed high peak abundance with even 15 μL of benzoylchloride. Because benzoylchloride is a non-volatile reagent with high boiling point (b.p. 197.2 °C), it is difficult to remove completely after the derivatization reaction. The residue of benzoylchloride also causes broadening of the chromatographic peak area, so 5 μL of benzoyl chloride was selected as an optimized amount.

Extraction Solvent. Liquid-liquid extraction (LLE) exploits the differences in the relative solubility of the analytes in two immiscible liquid phases. The selectivity and efficiency of the analyte is highly dependent on the choice of the two immiscible solvents in the extraction process. In this experiment, an extraction solvent was used to extract derivatized COPs from pyridine and benzoylchloride solution. The other conditions were fixed to conditions described in the section for the derivatization reaction and solvent extraction. The various solvents which differ with regard to polarity and solubility for the analytes, were used as an extraction solvent, and compared with regard to the peak area obtained from HPLC/UV-Vis.

The polarity of solvents is as follows: polarity, ethyl acetate > chloroform > methylene chloride > diethyl ether > toluene > hexane. As shown in Figure 9, ethyl acetate, methylene chloride, and chloroform, which have relatively high polarity, had increased extraction efficiency, and ethyl acetate showed the highest extraction efficiency among the five analytes.

Method Validation. The developed method was evaluated for the limit of detection (LOD), limit of quantification (LOQ), linearity of the calibration curve, accuracy, and precision. After sample preparation by an optimized experimental process, method validation was performed with spiked powder milk. The LOD was assumed from the instrumental analysis of a standard solution, and seven samples were prepared at a concentration within three times the assumed LOD. After the prepared samples were analyzed according to the established method, the standard deviation (σ) and the slope (m) of the linear calibration curve were calculated. The LOD and LOQ were set to 3σ/m and 10σ/m, respectively.

Table 1. LOD, LOQ, accuracy and precision for COPs from powdered milk

Compounds	LOD[a] (mg/kg)	LOQ[b] (mg/kg)	Conc. (mg/kg)	Accuracy[c] (recovery %)	RSD (%) (n=3)	Linear equation	Correlation Coefficient (r^2)
20α-Hydroxy cholesterol	0.039	0.13	0.13	105.8	5.0	y=2082x-208.1	0.999
			0.25	116.7	7.3		
			0.50	101.7	8.0		
			1.00	95.3	3.8		
			2.00	100.1	1.1		
			4.00	100.2	3.0		
7-Ketocholesterol	0.018	0.059	0.059	78.1	1.6	y=2527x-14.79	0.999
			0.10	104.6	5.4		
			0.20	103.2	1.2		
			0.40	100.6	2.6		
			0.80	100.4	2.1		
			1.60	99.9	1.2		
Cholestane-3β-5α-6β-triol	0.24	0.81	0.81	96.1	7.4	y=98.14x-13.94	0.996
			1.60	87.4	9.9		
			3.20	90.3	4.0		
			6.40	98.5	2.9		
			12.80	108.4	4.8		
			25.60	98.2	5.2		
5,6α-Epoxy cholesterol	0.11	0.38	0.38	113.0	2.8	y=293.73x-28.75	1.00
			0.80	97.0	5.0		
			1.60	95.9	5.9		
			3.20	104.6	3.0		
			6.40	97.8	3.5		
			12.80	100.3	2.4		
25-Hydroxy cholesterol	0.55	1.84	1.84	107.0	2.1	y=38.23x-208.1	0.999
			3.60	101.9	3.7		
			7.20	100.7	3.2		
			14.40	100.3	3.7		
			28.00	98.2	4.2		
			56.00	100.4	3.6		

[a]LOD (limits of detection): 3σ/m. [b]LOQ (limits of quantitation): 10σ/m. [c]Accuracy (recovery %) = (Measured value/Theoretical value) × 100

As a result, the LOD and LOQ of the five COPs from the spiked powder milk were in the concentration ranges of 0.018-0.55 mg/kg and 0.059-1.84 mg/kg, respectively. Although the LOD and LOQ were higher than in the LC/MS/MS method, because the COPs are found at a sub-ppm (μg/g) concentration level, the method can be used to determine the presence of COPs in dairy products.

The developed method for the simultaneous analysis of COPs by HPLC/UV-Vis is effective and sensitive method compared to the previous method which the limit of quantitation was 40-500 mg/kg.[24] The established derivatized method of cholesterol and COPs for HPLC/UV-Vis proved that reliable quantitation of COPs at the typical level (in the range of ppb (μg/kg) or ppm (mg/kg)) found the egg yolk,[16] milk and other dairy products[26,27] is possible.

The accuracy (as a relative recovery) and the precision (as RSD%) of the COPs in the concentration range of 0.059-56 mg/kg in the spiked powder milk were 78.1-116.7% and 1.1-9.9%, respectively. The results were fitted for the recommended acceptable values of −30% to +20% at each concentration level (Table 1).

To determine the residual COPs in a powdered milk sample, the samples were spiked with a standard solution of 0.059-56 mg/kg, and then the analytes were analyzed by an established analytical method. The calibration curves were obtained from the external standard method, and the corresponding correlation coefficients were above $r^2 = 0.996$ (Table 1).

Conclusions

As the UV spectra of cholesterol and COPs showed the maximum absorbance near 206 nm, and the absorbance wavelength of the mobile solvents (water and acetonitrile) in HPLC/UV-Vis may overlap, HPLC/UV-Vis is not suitable for analyzing COPs. Therefore, an effective derivatization/extraction method to increase the sensitivity for HPLC/UV-Vis was established. Five derivatized COPs showed good separation for quantification in a powdered milk sample using a gradient elution reversed-phase HPLC/UV-Vis

system. Also, the structures of the derivatized substances were confirmed using GC/MS. The established method of COPs for HPLC/UV-Vis analysis can be used as an alternative method.

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