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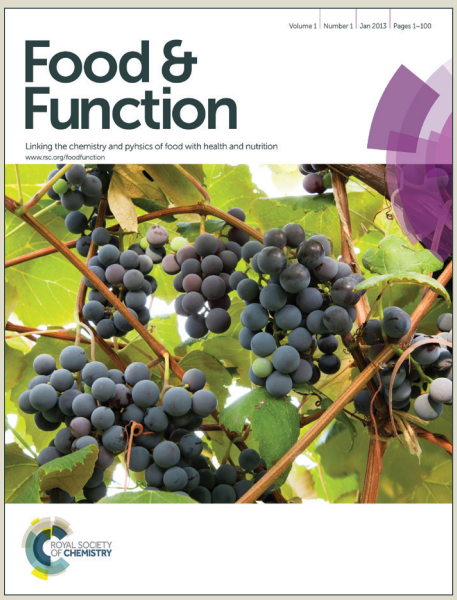


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# Cholesterol side chain analogs but not its ether analogs possess cholesterol-lowering activity

Lin Lei,[a] Xiaobo Wang,[a] Weihuan Huang,[b] Yuwei Liu,[a] Fangrui Zheng,[c] Ka Ying Ma,[a] Yuk Man Li,[a] Lijun Wang,[a] Sun Wa Man,[a] Chengnan Zhang,[a] and Zhen-Yu Chen*[a]

[a]Food & Nutritional Sciences Programme, School of Life Sciences, The Chinese University of Hong Kong, Shatin, NT, Hong Kong, China

[b]Institute of Traditional Chinese Medicine and Natural Products, Jinan University, Guangzhou, China

[c]Department of Chemistry, Chinese University of Hong Kong, Shatin, NT, Hong Kong, China

* Corresponding author: Dr. Zhen-Yu Chen
School of Life Sciences
The Chinese University of Hong Kong
Shatin, NT, Hong Kong, China
Tel: (852) 3943-6382
Fax: (852) 2603-7246
E-mail: zhenyuchen@cuhk.edu.hk

**Abstract**

Cholesterol analogs can be used to treat hypercholesterolemia. The present study was to test the effects of cholesteryl 3β-ethoxy (CE) and cholesteryl 3β-methoxy (CM) on plasma total cholesterol (TC) compared with that of β-sitosterol (SI) in hamsters fed a high cholesterol diet. CM and CE are the methoxy and ethoxyl analogs of cholesterol while SI is an analog of cholesterol having an additional ethyl group on the side chain. Results showed that SI at a dose of 0.1% could effectively reduce plasma TC by 18%. The analysis of sterols in plasma and liver did not detect the presence of SI, proving it was poorly absorbed in the intestine. In contrast, both CE and CM had no effect on plasma TC. However, CE and CM were found to accumulate in both plasma and liver, indicating they could be well absorbed in the intestine. It was therefore concluded that analogs having the different side chains possessed plasma TC-lowering activity, while analogs or derivatives on the hydroxyl group had no hypocholesterolemic activity.

**Keywords**

β-sitosterol, cholesteryl 3β-ethoxy, cholesteryl 3β-methoxy, cholesterol.

## Introduction

Coronary heart disease is the number one killer in the world with atherosclerosis being regarded as its major cause.[1] Atherosclerosis is an artery disease in which plaque builds in artery and thus reduces blood flow. The plaque consists mainly of cholesterol, fat, calcium and other substances. In this regard, elevation of plasma total cholesterol (TC), namely hypercholesterolemia, increases the risk of coronary heart disease.[2] In general, two types of medications, namely HMG-CoA reductase inhibitors and anion exchange resins, are effective in reducing plasma TC with the former inhibiting the synthesis of cholesterol in the liver while the latter inhibiting the absorption of cholesterol in the intestine.[3,4] In view of the well-reported side effects associated with the use of HMG-CoA reductase inhibitors and anion-exchange resins,[5,6] there has been always pressing need in the search of natural cholesterol-lowering compounds with higher efficacy and minimal side effects.

Phytosterols are actually analogs of cholesterol. In recent years, phytosterols as a healthy supplement have been widely used to treat the hypercholesterolemia.[7,8] β-Sitosterol (SI) is the major phytosterol present in human diet and has a structure similar to that of cholesterol (Figure 1). As an analog of cholesterol, SI has an additional ethyl group on the side chain at C-24 position. Due to its structural similarity with cholesterol, SI can impair the cholesterol absorption by competing with cholesterol for incorporation into the mixed micelles, and by displacing cholesterol from bile in the small intestine, thus leading to reduction in plasma TC. Most importantly, cholesterol absorption can reach more than 50%, while SI absorption is less than 5%.[3,4]

To our curiosity, we sought to ascertain if other analogs of cholesterol might also possess plasma TC-lowering activity. Therefore, the present study was to (i) synthesize two cholesterol ether analogs or derivatives, namely cholesteryl 3β-ethoxy (CE) and cholesteryl 3β-methoxy (CM) (Figure 1); and (ii) test if CE and CM could decrease plasma TC compared with SI.

## Materials and methods

### Synthesis of CE and CM

Methyl iodide (MeI), ethyl iodide (EtI), sodium hydride (NaH), tetrahydrofuran (THF), and dichloromethane were obtained from Guanghua Chemical Co. Ltd (Guangdong, China). THF (analytical reagent) was purified by refluxing over sodium and freshly distilled before use. CE and CM were synthesized following the method described previously.[9] In brief, cholesterol (12.95 mmol) was dissolved in freshly distilled THF (40 mL) followed by adding NaH (19.42 mmol) and 14.09 mmol of MeI or EtI. The reaction mixture was maintained at 60 $^{o}$C for 24 h, stopped by adding the distilled water (40 mL), and extracted with n-hexane (10 mL). The upper layer was washed with $H_2O$, dried on anhydrous MgSO4, and evaporated to obtain the crude CE or CM. The crude products were separated on a silica column. The fraction eluted by hexane-ether (v/v, 20:1) was evaporated to give the CE or CM. Structures of synthesized CE and CM were confirmed using NMR (Supplementary Figures 1-3). The GC analysis found that the purity of CE and CM was more than > 95%.

### Diets

Four diets were prepared as we previously described with some modifications (Table 1).[10] The control diet (CTL) was prepared by mixing all powdered ingredients (g): cornstarch, 508; casein, 242; lard, 50; sucrose, 119; mineral mix, 40, vitamin mix, 20; DL-methionine, 1; and cholesterol, 1. The three experimental diets were similarly prepared with adding 0.1% SI, 0.1 % CE, and 0.1 % CM, respectively, into the control diet. All four diets had 63.8, 24.6, and 11.5 % energy from carbohydrate, protein, and fat, respectively. The total cholesterol in diets was actually 1.045 mg/kg as lard contained 90 mg cholesterol/100 g.

### Hamsters

Male Golden Syrian hamsters (2.5 months) were divided into four groups (n = 10 each) fed the control, or one of three experimental diets for 6 weeks. Hamsters (n = 2

per cage) were housed in an animal room at 23 °C with 12/12-h light–dark cycles. Fresh diets were given daily and uneaten food was discarded. Food consumption was recorded daily, body weight was measured twice a week, and feces were collected weekly. They were allowed freely access to food and water. Blood sample was collected from the retro-orbital sinus under light anesthesia using a mixture of ketamine, xylazine, and saline (vol/vol/vol; 4:1:5) after overnight fasting at week 0 and 6. At the end of week 6, hamsters were sacrificed by $CO_2$. Livers were removed, washed with saline, weighed, and frozen in liquid nitrogen. All liver samples were stored at a -80 °C freezer before analysis. Experiments were approved and performed under the guidelines of the Animal Experimental Ethical Committee, The Chinese University of Hong Kong.

**Analysis of plasma lipids**

Commercial enzymatic kits from Infinity (Waltham, MA, USA.) and Stanbio Laboratories (Boerne, TX, USA) were used to determine plasma TC and total triacylglycerols (TG), respectively. Low-density lipoprotein cholesterol (LDL-C) and very low-density lipoprotein cholesterol (VLDL-C) were precipitated with phosphotungstic acid and magnesium chloride using a commercial kit (Stanbio Laboratories, Boerne, TX, USA). High-density lipoprotein cholesterol (HDL-C) in the supernatant was analyzed similarly as TC.[11] Non-HDL-C was calculated by deducting HDL-C from TC.

**Analysis of liver and plasma sterol**

Total liver and plasma sterol was determined as previously described.[12] In brief, lipids of liver and plasma were extracted using chloroform–methanol (2:1, v/v) with addition of 5α-cholestanol (1.0 mg) as an internal standard, followed by saponification. The sterols in the non-saponified fraction were converted to their TMS derivatives and then subjected to GC analysis on a fused silica capillary column (SAC$^{TM}$-5, 30 m × 0.25mm, i.d.; Supelco, Inc., Bellefonte, USA) in a Shimadzu GC-14B GC equipped with a flame ionization detector (Tokyo, Japan).

### Analysis of fecal neutral and acidic sterols

Feces were freeze-dried, grounded, and thoroughly mixed before analysis. Both fecal neutral and acidic sterols were measured as previously described.[13] To the fecal sample (300 mg), 5α-cholestane (0.5 mg) was added as an internal standard for quantification of total neutral sterols, while hyodeoxycholic acid (0.6 mg) was added as an internal standard for quantification of total acidic sterols. The samples were then saponified; the total neutral sterols were extracted with cyclohexane and converted to their TMS derivatives before GC analysis. Total acidic sterols in the aqueous phase were similarly converted to their TMS derivatives before GC analysis.

### Statistical Analysis

Data were expressed as mean ± standard deviation (SD). All data was analyzed with one-way analysis of variance (ANOVA) followed by Fisher's LSD test. Significance was defined as $P$ value < 0.05.

## Results

### Food intake, body and organ weights

All hamsters had an average food intake of about 8 g/day and a body weight gain of 11-14 g during the experimental period. However, there were no significant differences in food intake, body weight, and liver weights among the four groups (Data not shown).

### Plasma TC, HDL-C, non-HDL, non-HDL/HDL, HDL/TC and TG

Four groups of hamsters had similar lipoprotein profiles at the beginning of the experiment (Table 2). When the experiment reached the end of week 6, SI group had plasma TC, non-HDL-C and TG significant lower than the control, CE and CM groups. In contrast, plasma TC, non-HDL-C and TG in CE and CM groups were not significantly different from those in the control hamsters. Similarly, SI group had a lower ratio of

non-HDL-C to HDL-C but a higher ratio of HDL-C to TC compared with the control hamsters. In contrast, addition of CE and CM into diets had no effect of these ratios compared with those in the control group (Table 2).

**Plasma CE, CM and cholesterol**

At the end of week 6, SI group had liver cholesterol significantly lower than the other three groups ($p<0.05$, Table 2). In contrast, liver cholesterol concentrations in CE and CM groups were not significantly different from that in the control group. CE was detected in both plasma and liver of CE group, while CM was also detected in both plasma and liver of CM group (Figure 2 and Table 2). No SI was detected in both plasma and liver of SI group (Figure 2).

**Fecal total sterols**

The fecal sterols were separated into neutral and acidic groups. The neutral sterols consist of mainly cholesterol, coprostanol, coprostanone and dihydrocholesterol with the latter three being the metabolites of microbial fermentation in the large intestine. It is evident that SI group had greater excretion of coprostanol, cholesterol and dihydrocholesterol compared with the other three groups (Table 3, Figure 3). Addition of CE and CM into diets had no effect on the fecal excretion of total neutral sterols compared with that in the control hamsters (Table 3).

Fecal bile acids were separated into primary and second bile acids with the former being synthesized by the liver while the latter being the metabolites from bacterial actions in the colon. The primary bile acids include cholic and chenodeoxycholic, while the secondary ones were lithocholic and deoxycholic acids. Fecal analysis showed that SI group but not CE and CM groups significantly increased the excretion of the primary bile acids compared with the control group (Table 3). However, CE and CM groups but not SI group had the greater excretion of lithocholic acid compared with the control hamsters. As a whole, the three experimental groups significantly increased the fecal excretion of total bile acids compared with the control (Table 3).

## Discussion

The present study was to compare plasma TC–lowering activity of two types of cholesterol analogs, SI versus CE and CM, using hamsters as a hypercholesterolemic model. To the best of our knowledge, CE and CM do not exist in nature. We are not aware of any report that has investigated how SI, CE and CM affect differently plasma TC and cholesterol absorption. As shown in Figure 1, SI is structurally similar to cholesterol by having the same four rings and one hydroxyl group, but having a different side chain. Results showed that SI could decrease plasma TC by 18% ($p<0.05$) in hamsters fed a 0.1% cholesterol diet. In contrast, CE and CM had no effect on plasma cholesterol. Regarding other sterols having a different side chain with cholesterol, stigmasterol has also been shown to equally reduce plasma TC as SI.[14,15] The present results suggested that only cholesterol analogs having different chains (SI and stigmasterol) but not its ether analogs (CE and CM) possessed the plasma TC-lowering activity.

It has been shown that SI is poorly absorbed and its absorption rate is less than 5%.[3,4] This was confirmed in the present study. First, no detectable SI was found in the plasma and liver in the hamster fed the SI diet. Second, a large quantity of SI was seen in the feces (Table 3 and Figure 3), proving that the absorption of SI in the intestine was very minimal. It has been reported that compared with that on cholesterol, the different side chains render SI and other plant sterols being poorly absorbed in the intestine.[3,4] It was speculated that SI and other plant sterols were discriminated from incorporation into chylomicrons by intestinal acyl-CoA: cholesterol acyltransferase 2 (ACAT2). As an important enzyme involved in cholesterol absorption, function of ACAT2 is to convert free cholesterol to cholesteryl ester before cholesterol can be packed into chylomicrons. It has been shown that ACAT2 prefers free cholesterol to SI for esterification, thus discriminating SI from absorption.[16] In contrast to SI, CE and CM were better absorbed as they have the same side chains as that on cholesterol. First, it was observed that large amounts of CE and CM were present in plasma and liver of hamsters fed the diets containing CE

and CM (Table 2 and Figure 2). To be specific, the concentrations of CE and CM in the liver reached 23-24 mg/g. Second, the fecal analysis also found that CE and CM hamsters daily excreted about 1.15-1.34 mg CE or CM, which was comparable to excretion of 1.09-1.11 mg cholesterol and its microbial metabolites. The present study clearly demonstrated that the absorption behavior of CE and CM was similar to that of cholesterol but it was different from that of SI.

## Conclusion

We investigated the effects of two types of cholesterol analogs on plasma TC in hypercholesterolemia hamsters. It was concluded that the side chain analog, SI, was effective in reducing plasma TC, while the ether analogs, CE and CM, had no such activity. It was evident that CE and CM were well absorbed in the intestine, while SI was not or poorly absorbed. The future searching for potential hypocholesterolemic sterols shall focus on the analogs having different side chains but not on ones having derivations on the rings.

## Acknowledgment

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**Table 1** Composition of the control (CTL) and three experimental diets containing 0.1% β-sitosterol (SI), 0.1% cholesteryl 3β-ethoxy (CE), and 0.1% cholesteryl 3β-methoxy (CM), respectively

| Ingredients (g) | CTL | SI | CE | CM |
|---|---|---|---|---|
| Corn starch | 508 | 508 | 508 | 508 |
| Casein | 242 | 242 | 242 | 242 |
| Sucrose | 119 | 119 | 119 | 119 |
| Lard | 50 | 50 | 50 | 50 |
| Mineral mixture | 40 | 40 | 40 | 40 |
| Vitamin mixture | 20 | 20 | 20 | 20 |
| Gelatin | 20 | 20 | 20 | 20 |
| DL-Methionine | 1 | 1 | 1 | 1 |
| Cholesterol | 1 | 1 | 1 | 1 |
| β-Sitosterol | — | 1 | — | — |
| Cholesteryl 3β-ethoxy | — | — | 1 | — |
| Cholesteryl 3β-methoxy | — | — | — | 1 |

**Table 2** Changes in plasma total cholesterol (TC), cholesteryl 3β-ethoxy, cholesteryl 3β-methoxy, triacylglycerols (TG), high-density lipoprotein cholesterol (HDL-C), non-HDL-C and liver sterol in hamsters fed the control (CTL) or one of three experimental diets containing 0.1% β-sitosterol (SI), 0.1% cholesteryl 3β-ethoxy (CE), and 0.1% cholesteryl 3β-methoxy (CM), respectively, for 6 weeks

| | CTL | SI | CE | CM | *P* value |
|---|---|---|---|---|---|
| **Week 0** | | | | | |
| TC (mmol/L) | 4.0±0.5 | 4.0±0.6 | 4.0±0.2 | 4.0±0.5 | 0.98 |
| TG (mmol/L) | 0.6±0.2 | 0.6±0.2 | 0.6±0.2 | 0.6±0.2 | 0.99 |
| HDL-C (mmol/L) | 2.8±0.2 | 2.8±0.2 | 2.7±0.1 | 2.7±0.2 | 0.64 |
| Non-HDL-C (mmol/L) | 1.1±0.4 | 1.2±0.4 | 1.3±0.1 | 1.3±0.5 | 0.77 |
| Non-HDL-C/HDL-C | 0.4±0.1 | 0.4±0.1 | 0.5±0.1 | 0.5±0.2 | 0.48 |
| HDL-C/TC | 0.7±0.1 | 0.7±0.1 | 0.7±0.1 | 0.7±0.1 | 0.51 |
| **Week 6** | | | | | |
| TC (mmol/L) | 6.2±0.7a | 5.1±1.0b | 6.2±0.9a | 6.2±0.7a | <0.01 |
| TG (mmol/L) | 2.6±1.2a | 1.3±0.2b | 2.3±0.8a | 2.3±0.6a | <0.01 |
| HDL-C (mmol/L) | 3.5±0.4 | 3.7±1.1 | 3.4±0.2 | 3.4±0.3 | 0.71 |
| Non-HDL-C (mmol/L) | 2.7±0.4a | 1.4±1.1b | 2.9±0.7a | 2.8±0.4a | <0.01 |
| Non-HDL-C/HDL-C | 0.8±0.1a | 0.5±0.4b | 0.9±0.2a | 0.8±0.1a | <0.01 |
| HDL-C/TC | 0.6±0.1b | 0.7±0.2a | 0.6±0.1b | 0.6±0.1b | <0.01 |
| Liver cholesterol (mg/g) | 33.4±5.6a | 29.6±6.3b | 37.9±5.1a | 37.0±3.1a | <0.01 |
| Cholesteryl 3β-ethoxy | | | | | |
| Plasma (mg/L) | — | — | 72.1±6.1 | — | — |
| Liver (mg/g) | — | — | 24.0±2.3 | — | — |
| Cholesteryl 3β-methoxy | | | | | |
| Plasma (mg/L) | — | — | — | 131.7±21.5 | — |
| Liver (mg/g) | — | — | — | 23.3±0.7 | — |

Data were expressed as mean ± SD. [a,b] Mean values in a row with different letters differ significantly ($p < 0.05$).

**Table 3** Daily fecal excretion of neutral and acidic sterols in hamsters fed the control (CTL) or one of the three experimental diets containing 0.1% β-Sitosterol (SI), 0.1% cholesteryl 3β-ethoxy (CE), and 0.1% cholesteryl 3β-methoxy (CM), respectively, for 6 weeks

| | CTL | SI | CE | CM | *P* value |
|---|---|---|---|---|---|
| Neutral sterols (mg/day) | | | | | |
| Coprostanol | 0.43±0.08b | 1.01±0.32a | 0.43±0.06b | 0.41±0.03b | <0.01 |
| Coprostanone | 0.06±0.01b | 0.07±0.05b | 0.05±0.01b | 0.15±0.06a | <0.01 |
| Cholesterol | 0.27±0.05c | 1.02±0.13a | 0.48±0.16b | 0.38±0.08b | <0.01 |
| Dihydrocholesterol | 0.17±0.02b | 0.68±0.13a | 0.15±0.01b | 0.14±0.03b | <0.01 |
| Total | 0.94±0.15b | 2.78±0.62a | 1.11±0.11b | 1.09±0.09b | <0.01 |
| | | | | | |
| β-Sitosterol | — | 3.54±0.64 | — | — | — |
| Cholesteryl 3β-ethoxy | — | — | 1.34±0.11 | — | — |
| Cholesteryl 3β-methoxy | — | — | — | 1.15±0.10 | — |
| | | | | | |
| Acidic sterols (mg/day) | | | | | |
| Lithocholic acid | 1.14±0.21b | 0.78±0.23c | 1.73±0.45a | 2.00±0.38a | <0.01 |
| Deoxycholic acid | 0.01±0.00b | 0.25±0.07a | 0.02±0.01b | 0.02±0.01b | <0.01 |
| Chenodeoxycholic acid+Cholic acid | 0.08±0.05b | 0.95±0.37a | 0.13±0.06b | 0.14±0.07b | <0.01 |
| Total | 1.23±0.23b | 1.98±0.42a | 1.89±0.50a | 2.14±0.37a | <0.01 |

Data were expressed as mean ± SD. [a,b,c] Mean values in a row with different letters differ significantly ($p < 0.05$).

## Figure legends

Figure 1. Structures of cholesterol, β-sitosterol, cholesteryl 3β-ethoxy, and cholesteryl 3β-methoxy.

**Figure 2.** Gas chromatographic trace of liver sterols at week 6 in hamsters fed the control (CTL) and three experimental diets containing 0.1% β-Sitosterol (SI), 0.1% cholesteryl 3β-ethoxy (CE), and 0.1% cholesteryl 3β-methoxy (CM), respectively. Peak 1, 5α-cholestane (internal standard); 2, cholesterol; 3, cholesteryl 3β-ethoxy; 4, cholesteryl 3β-methoxy.

**Figure 3**. Gas chromatographic trace of fecal neutral sterols at week 6 in hamsters fed the control (CTL) and three experimental diets containing 0.1% β-Sitosterol (SI), 0.1% cholesteryl 3β-ethoxy (CE), and 0.1% cholesteryl 3β-methoxy (CM), respectively. Peak 1, 5α-cholestane (internal standard); 2, coprostanol; 3, coprostanone; 4, cholesterol; 5, dihydrocholesterol; 6, β-Sitosterol 7, cholesteryl 3β-ethoxy; 8, cholesteryl 3β-methoxy.

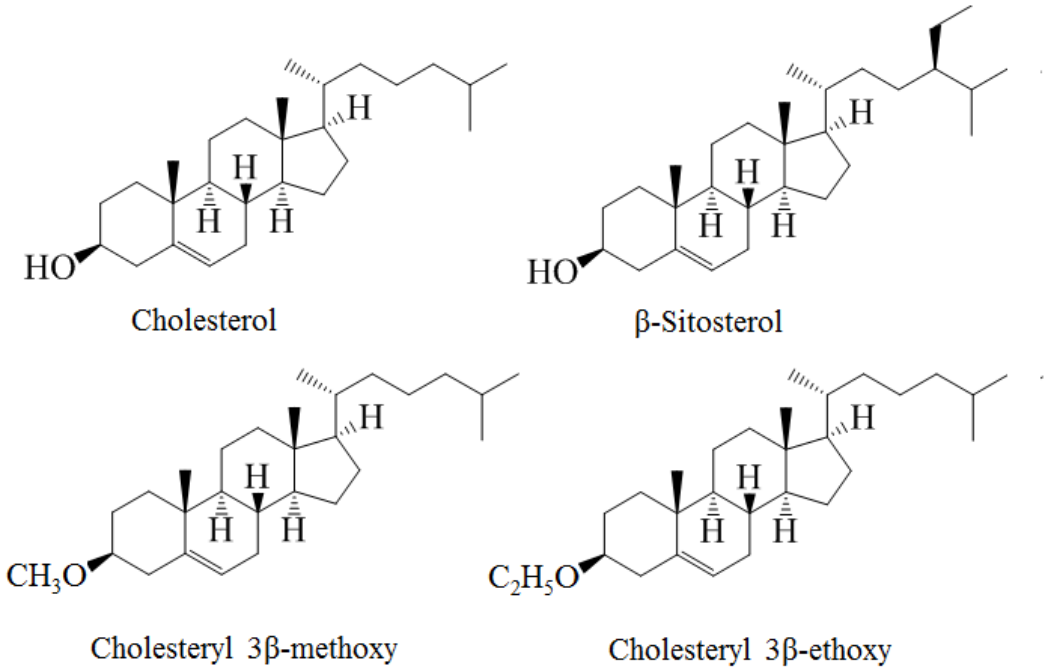



Figure 1

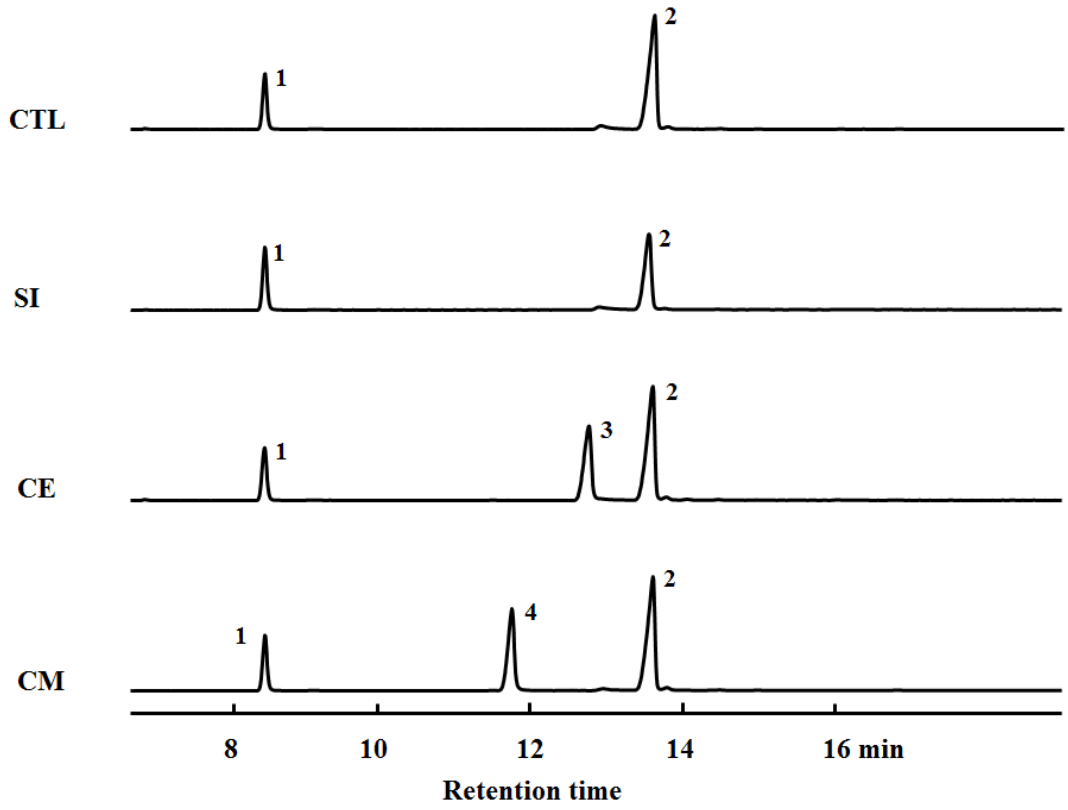



Figure 2

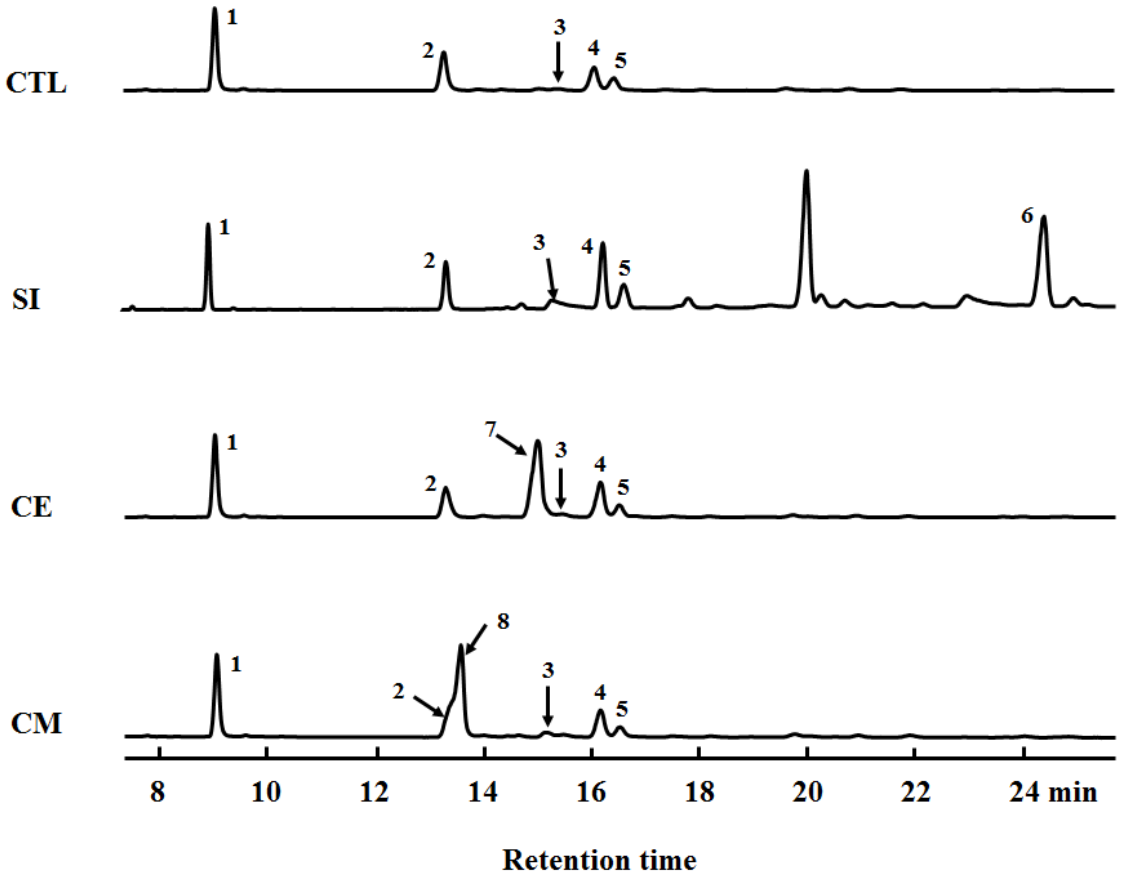
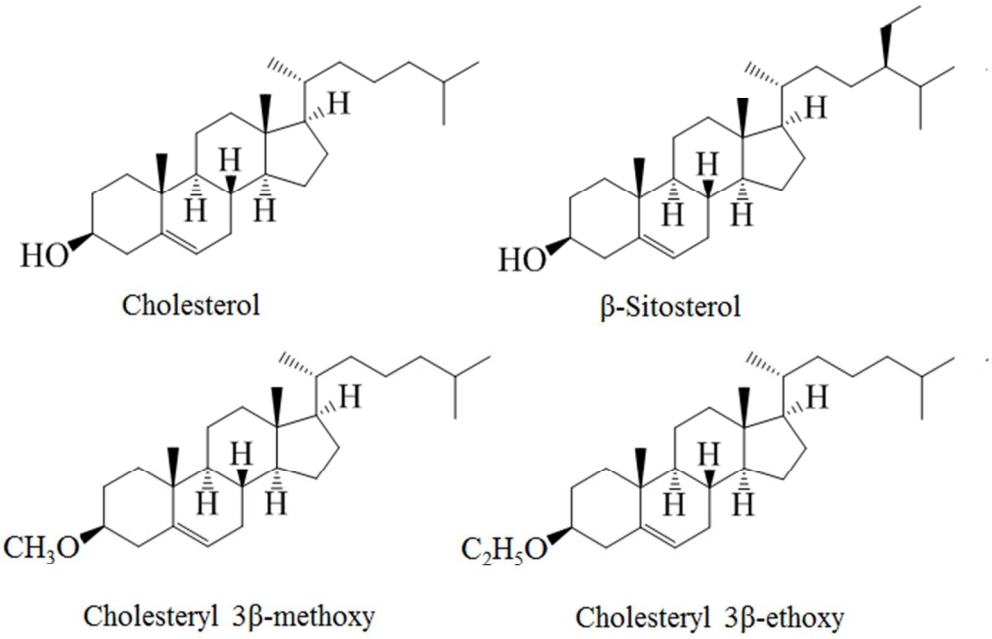



Figure 3

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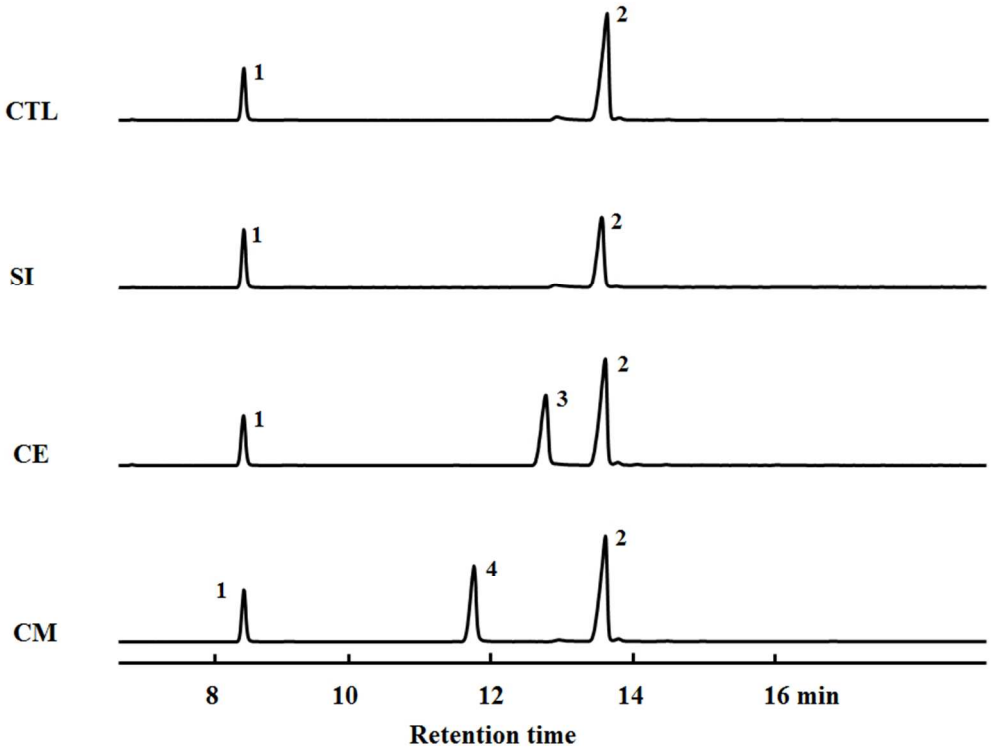


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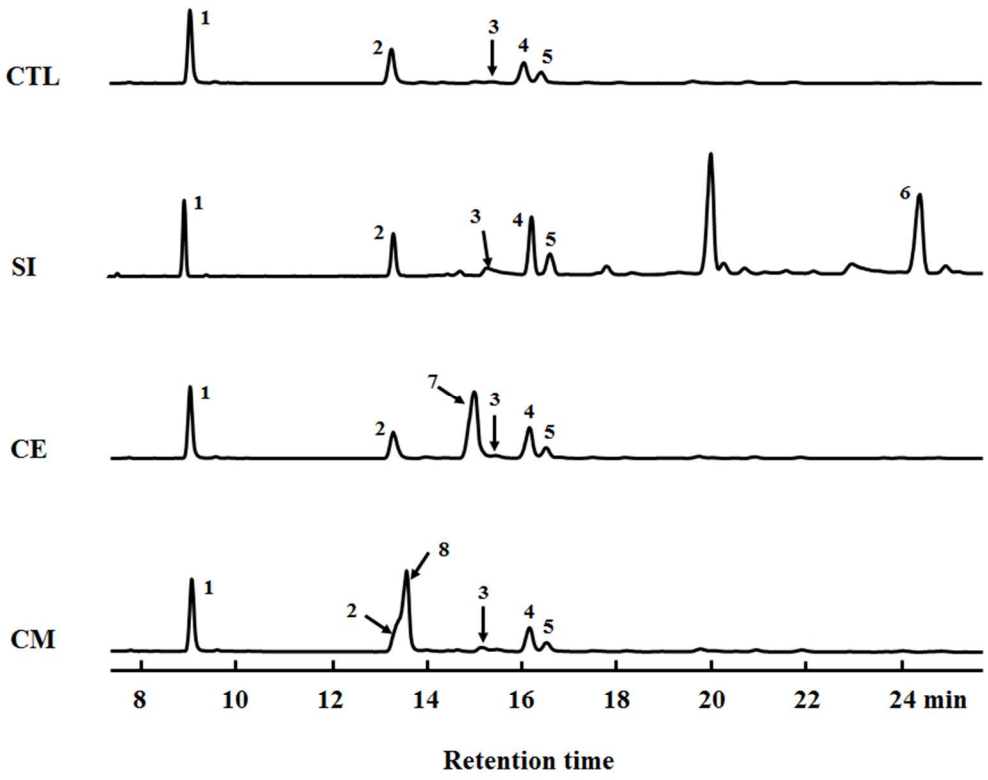


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