Supporting information for

Thiyl Radical-Based Charge Tagging Enables Sterol Quantitation via Mass Spectrometry

Sarju Adhikari[1,2] and Yu Xia[*1,2]

1. Department of Chemistry, Purdue University, West Lafayette, IN 47907, USA

2. Department of Chemistry, Tsinghua University, Beijing 100084, P.R. China

To whom correspondence should be addressed:

Prof. Yu Xia, xiayu@email.tsinghua.edu.cn

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1. Reagents and materials

All the reagents and solvents were purchased from commercial sources and were used without further purification. Cholesterol standard (purity ≥99%) was purchased from Nu-Chek Prep, Inc. (Elysian, MN, USA). Campesterol (purity >99%), and cholesterol-25,26,26,26,27,27,27-d_7 (purity >99%) were purchased from Avanti Lipids Polar, Inc. (Alabaster, AL, USA). β-sitosterol (purity >95%), stigmasterol (purity ~95%), brassicasterol (purity >98%), cycloartenol (purity ≥90%), *N,O*-bis-trimethylsilyltrifluoroacetamide (BSTFA) with 1% of trimethylchlorosilane (TMCS), *N,N*-dimethylformamide (DMF), cysteamine hydrochloride, Sodium 2-mercaptoethanesulfonate (MESNA), thioglycolic acid (TGA), 2,2-dimethoxy-2-phenylacetophenone (DMPA), sodium hydroxide (NaOH), ethanol, potassium hydroxide (KOH), *n*-hexane, diethyl ether, chloroform and ethyl acetate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Deionized water was obtained from a purification system at 0.03 µS·cm (model: Micropure UV; Thermo Scientific; San Jose, CA, USA). Pooled human plasma (Li Heparin used as anticoagulant) was purchased from Innovative Research (Novi, MI, USA). Vegetable oil (soybean oil) was bought from a supermarket and then stored at -20°C until analysis.

2. Experimental Procedures

2.1 Extraction protocols for sterols analysis from vegetable oil and human plasma

2.1.1 Total sterols in human plasma

The extraction procedure is derived as previously described by Yang et al.[1] An aliquot of human plasma (30 µL, spiked with 100 µL of cholesterol-d_7, 394 µg/mL in chloroform) and then saponified in 2 mL of 2 M KOH ethanol/water (8:2, v/v) at 80°C for 1 h. Saponification is performed to convert sterol esters to free sterols, and also to remove other lipids (fatty acids and glycerin) from the extract before sterol analysis. After cooling, 2 mL of deionized water was added, followed by 3 mL of *n*-hexane. The mixture was vortexed for 1 min and then, centrifuged for 5 minutes at 3000 x g. The upper hexane layer was collected, and the extraction procedure was repeated twice with *n*-hexane. The hexane extracts were combined and evaporated to dryness, dissolved in 1 mL chloroform and stored until derivatization process for analysis.

2.1.2 Esterified and free sterols in human plasma

Separation of esterified and free sterols in human plasma was determined following the method developed by Lund et al.[2] An aliquot of human plasma (30 µL, spiked with 100 µL of cholesterol-d_7, 394 µg/mL in chloroform) was subjected to Bligh/Dyer extraction, dried under nitrogen, and reconstituted in 1 mL toluene. The lipid extract was loaded onto a 100-mg Isolute silica cartridge (Biotage, Charlotte, NC) previously conditioned with 2 mL of *n*-hexane. Esterified sterols were eluted first with 2 mL of *n*-hexane, while free sterols eluted next with 8mL of 30% isopropanol in *n*-hexane. Both fractions of sterols were separately subjected to saponification, and extraction according to the procedures described for the total sterol analysis.

2.1.3 Total Sterols in vegetable oil

The procedure is derived from the extraction protocol as reported by Bedner et al.[3] 30 mg of the vegetable oil was spiked with 100 µL of internal standard (cholesterol-d_7, 394 µg/mL in chloroform) was subjected directly to saponification, and extraction according to the procedures for total sterol analysis for human plasma.

2.1.4 Esterified and free sterol in vegetable oil

Separation of esterified and free sterols in vegetable oil was determined following the extraction procedure previously reported by Olivera et. al.[4] 30 mg of the vegetable oil (spiked with 100 µL of cholesterol-d_7, 394 µg/mL in chloroform) was diluted with 1 mL of *n*-hexane and was loaded onto a 300-mg SPE silica cartridge (Biotage, Charlotte, NC) previously conditioned with 5 mL of *n*-hexane. Esterified sterols were eluted first with 10 ml of *n*-hexane/diethyl ether (95:5, v/v), while the more polar free sterols were extracted next using 10 mL of *n*-hexane/diethyl ether/ethanol (25:25:50, v/v/v). Both fractions of sterols were separately subjected to saponification, and extraction according to the procedures described for the total sterol analysis.

2.2 Derivatization of sterols in bulk processes

A mixture of sterol (0.1-200µM range), thiol reagent (100 mM), and DMPA (photo-initiator) (1 mM) was dissolved in DMF in a borosilicate scintillation glass vial. The content of the vial was mixed thoroughly to ensure uniformity and was degassed with nitrogen gas for 5 minutes. The mixture was then irradiated using a low-pressure mercury (LP-Hg) lamp with an emission band at 351nm (model number: 80-1057-01/351; BHK, Inc.; Ontario, CA, USA) for 15-20 minutes at room temperature as shown in Figure S2. This set-up, both the light source and the vial, was covered with aluminum foil, in order to increase the light intensity by reflecting UV light. All reactions were carried out at ambient temperature. The reaction progress can be monitored by MS. The reaction solution was then dissolved in ~0.1 N sodium hydroxide aqueous solution and was extracted with ethyl acetate (x 2) to remove the excess thioglycolic acid.

2.3 Derivatization of sterols in a photochemical microreactor

A schematic overview of the photochemical microreactor setup is shown in figure 1 (c). The flow reactor capillary (fused silica capillary tubing (100 µm i.d., 375 µm o.d.)) is passed through a small cardboard box (reaction chamber), without coiling. The overall length of the tube inside the chamber is 10 cm. The LP-Hg lamp (emission band at 351 nm) is placed next to capillary tube (at a distance of 0.5 cm) inside the box. All the reactant with the optimized reaction concentration from the bulk processes were premixed and degassed prior to introduction into the chamber and were irradiated in one-layer continuous flow format (µL/min rate). The total residence time for the reaction was calculated by the total reactor volume exposed to the UV irradiation by the sample flow rate. It is worth mentioning that the overall set-up of the photo-microreactor itself is quite general, and can be readily applied or optimized for other photochemical applications.

2.4 Mass spectrometry

All mass analysis of derivatized sterols were performed using nano-electrospray ionization (nanoESI)-MS. NanoESI tips (~10 µm o.d.) were made from borosilicate glass capillary tips (1.5 mm o.d. and 0.86 mm i.d.) using a micropipette puller (P-1000

Flaming/Brown; Sutter Instrument, Novato, CA, USA). Data collection were done on a 4000 QTRAP (triple quadrupole/linear ion trap (LIT)) mass spectrometer. Analyst software 1.6.2 (Sciex) was used for instrument control and data processing. MS^1 mass analysis were performed in LIT mode in Q3 by the mass selective axial scan.[5] Typical MS parameters during the study were: nanoESI spray voltage, - (1500-1800) V; curtain gas, 10 psi; declustering potential, -20V; scan rate, 1000 Da/s unless otherwise specified. Two modes of Collision-induced dissociation (CID) were performed: beam type CID and ion trap CID. For neutral loss scan (NLS), collision energy (CE) optimized at 32 V was used.

3. Results

3.1 Accurate Mass Measurements

Accurate mass measurements were performed on an LTQ Orbitrap XL mass spectrometer (Thermo Scientific, San Jose, CA) to acquire elemental composition of the reaction products. Figure S1(a) shows ESI MS^1 spectrum for TGA derivatized cholesterol. Deprotonated ^{TGA}cholesterol ($[M-H]^-$) was found at *m/z* 475.3253, having a chemical formula of $C_{29}H_{48}O_3S^-$ (relative error:1.4 ppm). The elemental composition of the derivatized products corresponds to a net addition of $C_2H_2O_2S$ to a cholesterol ($C_{27}H_{46}O$). Therefore, it is proposed that one molecule of thioglycolic acid ($HSCH_2CO_2H$) is added to the cholesterol. If a second thiyl radical addition happened, the product should have appeared at *m/z* 567.3178. Such a product was not observed. However, we found a small peak at *m/z* 549.3080 ($C_{31}H_{49}O_4S_2^-$, ppm error: 1.4), which could result from H_2O loss from the second addition product. Figure S1(b) shows ESI-MS/MS of deprotonated ^{TGA}cholesterol. A dominant fragment channel, corresponding to a neutral loss of 44 Da, is observed at *m/z* 431.3348. The elemental composition of this daughter ion corresponds to a chemical formula of $C_{28}H_{48}O_3S^-$, due to the loss of CO_2 from the carboxylate functional group of the TGA.

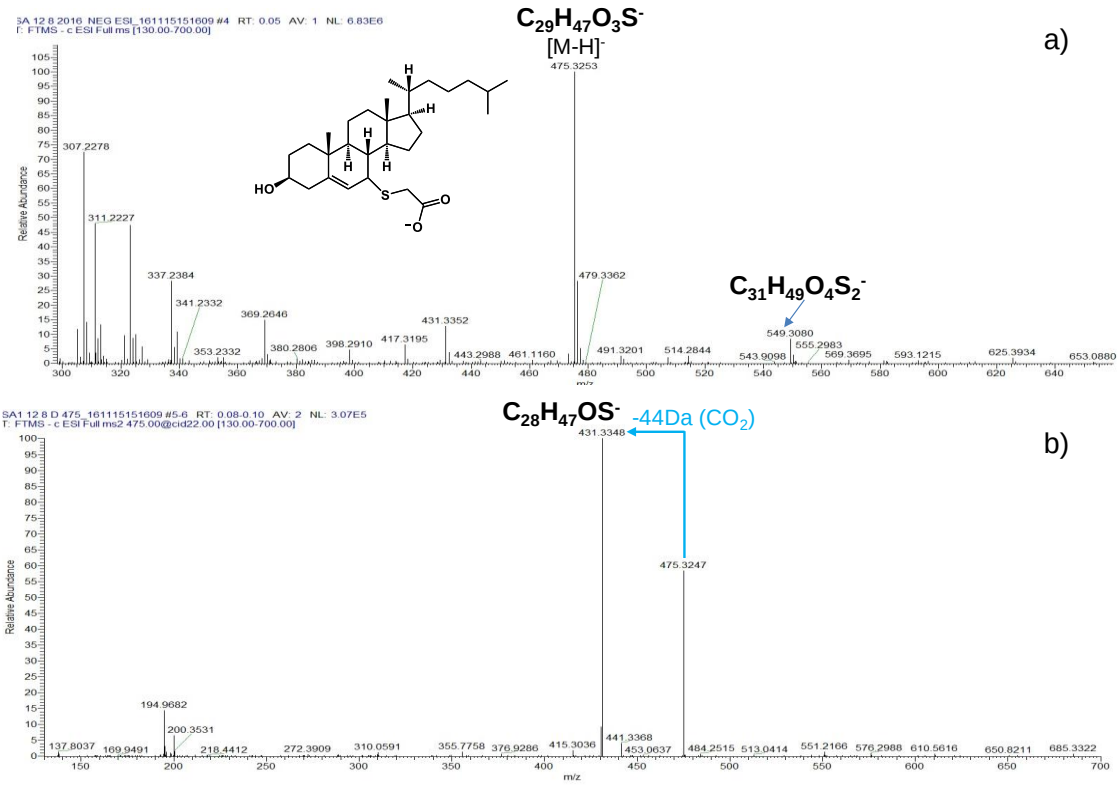

Figure S1. Accurate mass measurements for TGA derivatized cholesterol (a) ESI MS^1 in negative mode for ^{TGA}cholesterol detected at *m/z* 475.3253. (b) MS/MS of ^{TGA}cholesterol from panel (a).

3.2 Reaction kinetics for bulk reactions

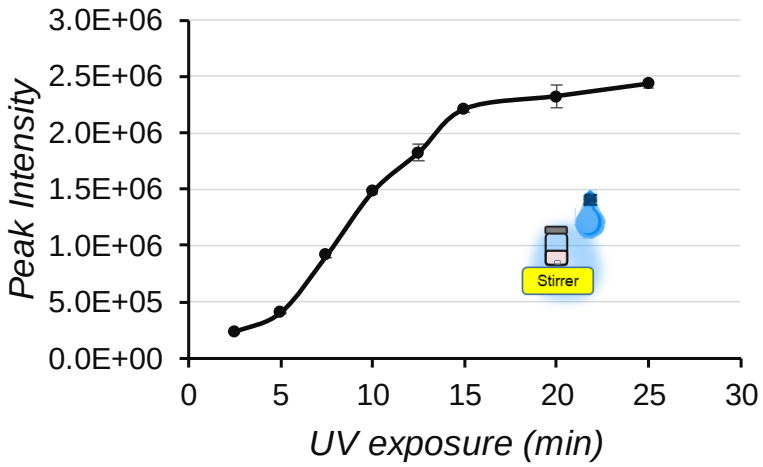

Figure S2. Reaction kinetics for TGA derivatization of cholesterol using batch processes plotted over UV exposure. The inset shows schematics for bulk reaction.

3.3 Derivatization yield for cholesterol

GC-MS analysis of sterols was performed to estimate the yield of derivatization. Cholesterol was derivatized using BSTFA+1%TMCS as the silylating reagent and was operated in the SIM mode. Analysis was carried out with a Shimadzu QP-2010 GC-MS system (70 eV, electron ionization mode), equipped with DB-5MS (20.0 m X 0.18 mm I.D., 0.18 µm film thickness) column. Helium was used as the carrier gas, at a flow rate of 1.0 mL/min. Samples were introduced in split-injection mode (1:20). The column oven temperature was set initially at 70.0 ^{o}C (3 min) and then programmed to rise to 325.0 ^{o}C (3 min) at a rate of 13 ^{o}C/min. The mass range scanned was *m/z* 50-800. The yield was determined based on the loss of the silylated cholesterol peak following the photochemical derivatization reaction.

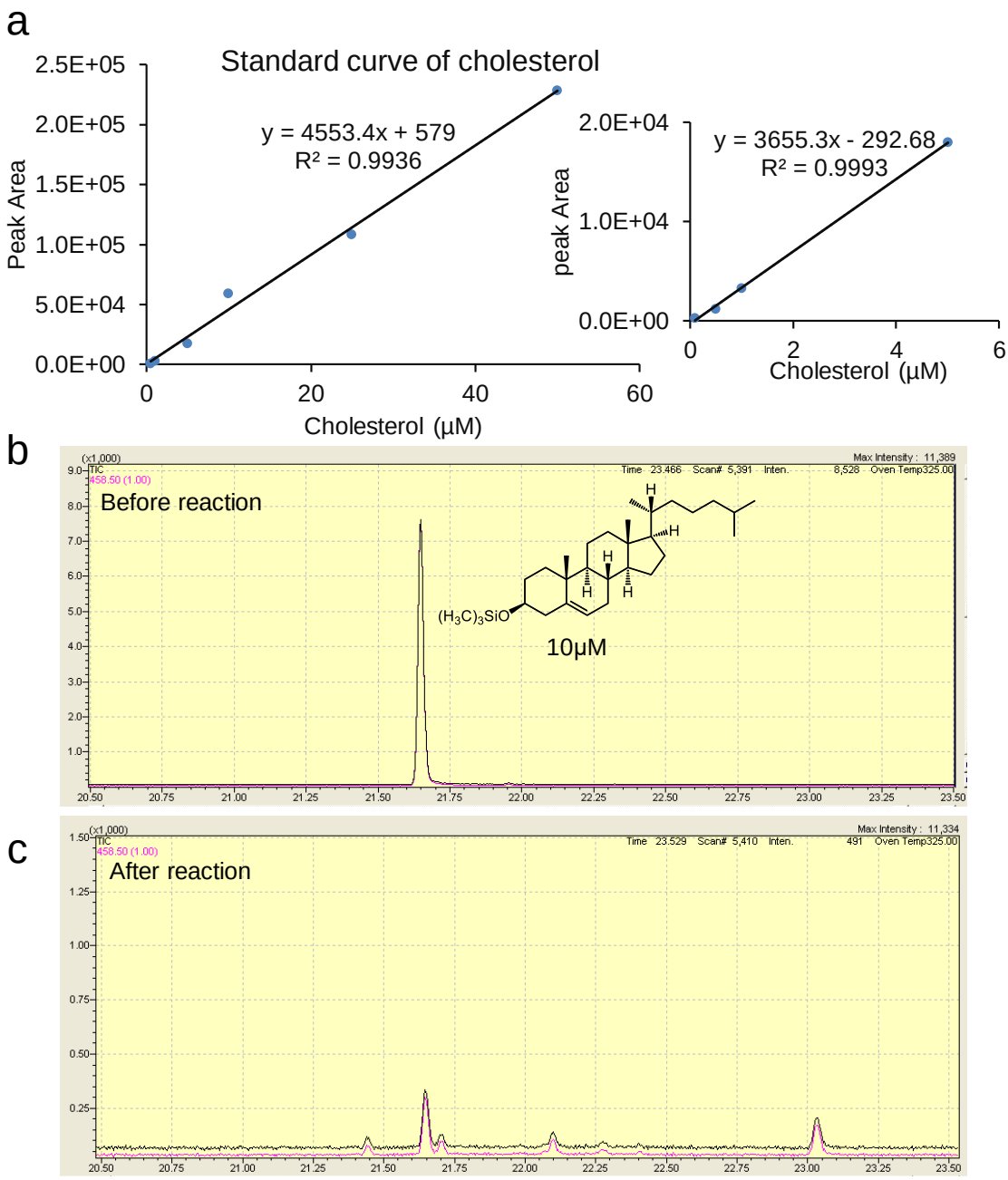

Figure S3. (a) GC/MS standard curve used for determination of the derivatization yield of Cholesterol. GC/MS chromatograms of silylated cholesterol (retention time 21.65 min) (b) before reaction (c) after reaction.

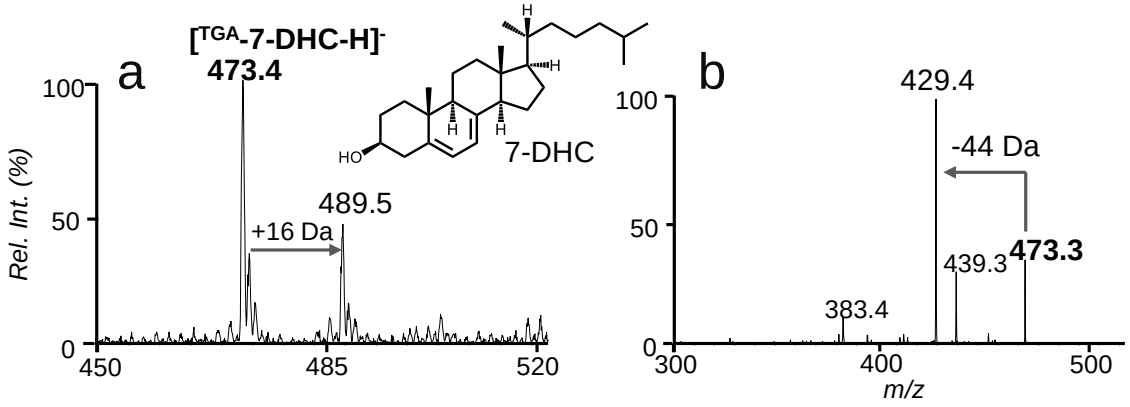

Figure S4. NanoESI-MS of TGA derivatized (a) 7-dehydrocholesterol (1 µM, detected at *m/z* 473.3, negative ion mode), (b) MS^2 CID of $[^{TGA}$-7-DHC-H$]^-$.

3.4 Neutral loss scan of ^{TGA}-cholesterol

Beam-type CID of ^{TGA}-cholesterol was performed under different collision energy (CE) ranging from 5 to 75 V. The dominant fragment ion (*m/z* 431.3, 44 Da NL) was monitored. The optimal CE for the 44 Da loss was 32 V, which was chosen for the 44 Da NLS experiments. Limit of detection for ^{TGA}-cholesterol was determined as the concentrations for which the signal response is 3 times of noise from blank in NLS.

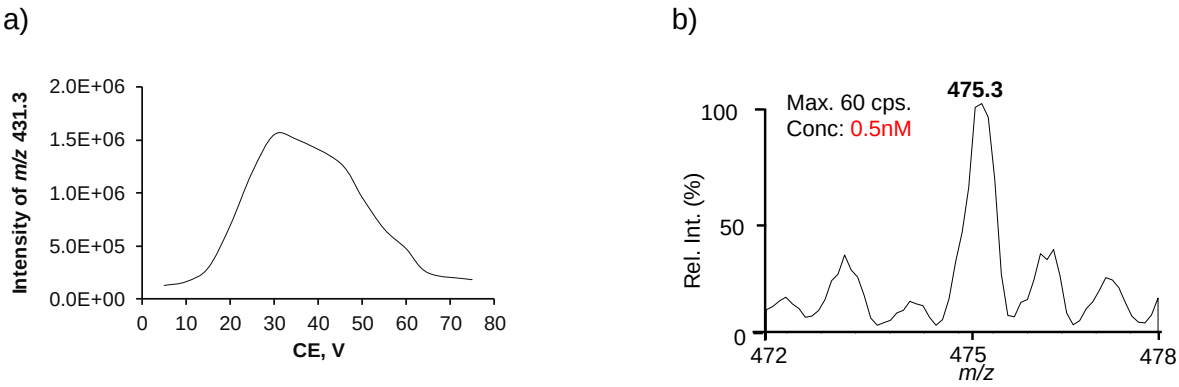

Figure S5. a) Plot intensity of fragment *m/z* 431.3 of ^{TGA}-cholesterol with varying rolling collision energy (CE), used to find optimal CE value for quantitative analysis and b) limit of detection for ^{TGA}-cholesterol (*m/z* 475.3) using NLS (44 Da loss). The spectrum was averaged for 3 minutes.

3.5 Beam-type CID mass spectra of TGA derivatized sterol standards

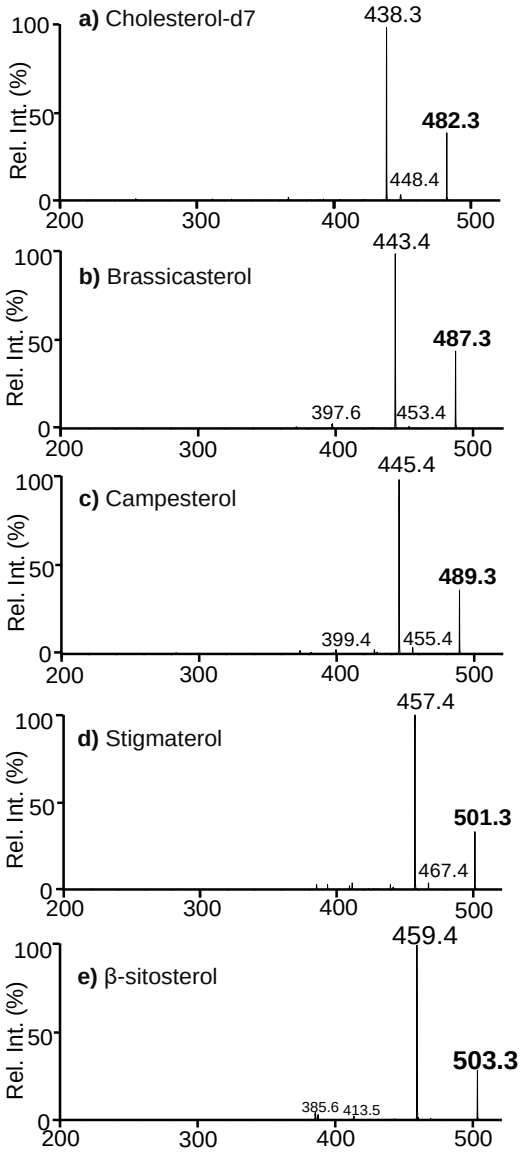

Figure S6: Beam-type CID mass spectra of TGA derivatized sterol standards (a) Cholesterol-d_7 (b) Brassicasterol (c) Campesterol (d) Stigmasterol (e) β-Sitosterol.

3.6 Analysis of sterols in complex lipid mixtures

3.6.1 Calibration curves for the sterol standards

Calibration curves for cholesterol, brassicasterol, campesterol, stigmasterol, and β-sitosterol were constructed based on NLS of 44 Da, using 5μM of cholesterol-d7 as internal standard. The TGA derivatized sterols gave excellent correlation coefficient (R^2) as shown in Table S1.

Table S1. Calibration curve equations for TGA derivatized sterol standards based on 44 Da NLS, using cholesterol-d_7 (5 μM) as the internal standard.

Sterols	Calibration curve equation	R^2
Cholesterol	$y = 0.6057x - 0.2806$	0.9968
Brassicasterol	$y = 0.2481x - 0.0120$	0.9959
Campesterol	$y = 0.3275x - 0.0701$	0.9943
Stigmasterol	$y = 0.2576x - 0.0114$	0.9993
β-Sitosterol	$y = 0.3544x - 0.0667$	0.9991

3.6.2 Recovery rate

Table S2: List of the recovery rates of several sterols tested in this study, [Mean ± SD] % (number of trials: 3).

Sterols	Free sterol	Total sterol
Cholesterol[a]	90 ± 5	100 ± 2
β-Sitosterol[b]	95 ± 5	98 ± 7
Stigmasterol[b]	98 ± 6	98 ± 4
Campesterol[b]	93 ± 7	96 ± 6
Brassicasterol[b]	94 ± 7	94 ± 6

[a]Data acquired from human plasma
[b]Data acquired from vegetable oil

3.6.3 Analysis of esterified and total sterol in human plasma and vegetable oil

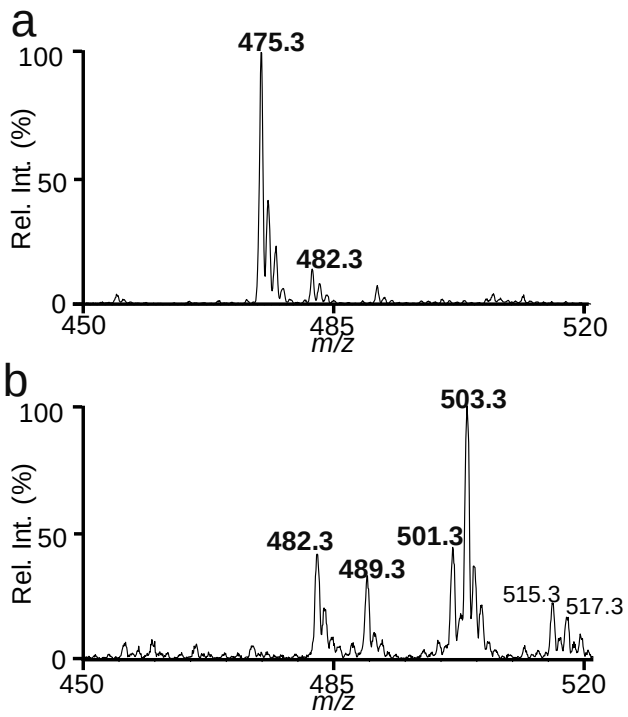

Figure S7: Analysis of sterols in biological samples. Negative ion MS^1 profile of derivatized sterols (with 5 µM Chol-d_7 as IS) recovered from (a) human plasma and (b) vegetable oil.

Table S3: Sterol species in human plasma based on ref (6), including corresponding exact mass and detection *m/z* (after derivatization with thioglycolic acid).

Sterol	Exact mass	Detection *m/z*
7-dehydrocholesterol	384.34	473
Desmosterol	384.34	473
Lathosterol	386.35	475
7-oxocholesterol	400.37	489
Campesterol	400.37	489
24S-hydroxycholesterol	402.35	491
25-hydroxycholesterol	402.35	491
27-hydroxycholesterol	402.35	491
4β-hydroxycholesterol	402.35	491
7α-hydroxycholesterol	402.35	491
Sitosterol	414.39	503
Lanosterol	426.39	515

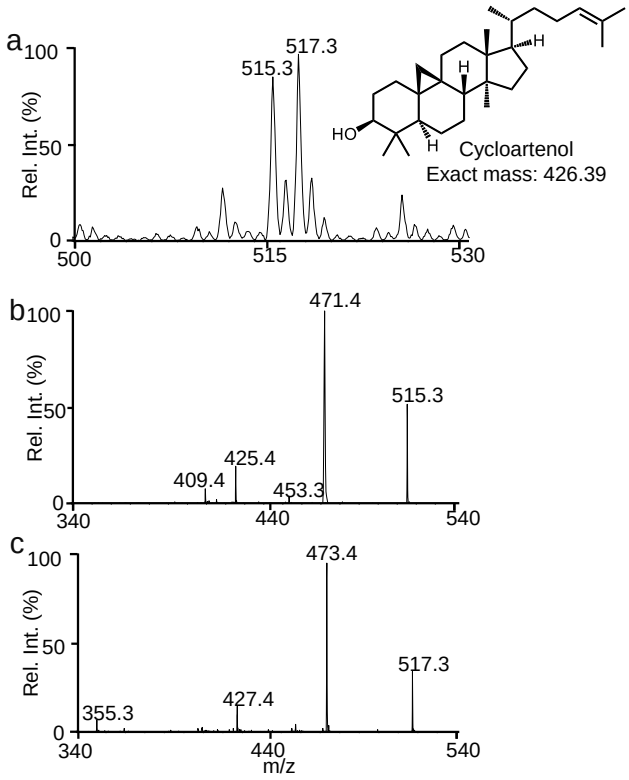

Figure S8: a) Negative ion nanoESI MS^1 spectrum of 5µM TGA-derivatized cycloartenol (b) MS/MS of *m/z* 515.3 (c) MS/MS of *m/z* 517.3

4. References

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