Extraction and Analysis of Sterol Lipids

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Synopsis: This protocol describes the standard method for the extraction and analysis of sterols following a LIPID MAPS time course protocol. Cells should be grown and treated according to protocol PP0000001003 or PP0000001800. Sterols are extracted via a modified Bligh-Dyer method and the sterol fraction purified using silica based solid phase extraction (SPE). After resolution, sterols are separated using a reverse phase binary liquid chromatography (LC) gradient and quantitated using a MRM method with positive electrospray ionization mass spectrometry (ESI-MS).

I. Extraction and Purification of Sterol Lipids

The extraction protocol outlined below is for cells grown in 60 or 100mm dishes suspended in 2ml of DPBS. After extraction, sterols are purified using SPE using cartridges from Isolute (Isolute Si, 10ml, 100mg, cat # 460-0010-G, Biotage). DNA is used to normalize sterol quantity.

Reagents Required:

Chloroform	High purity water
DPBS	Isolute 100mg silica SPE columns
EDTA	MeOH
EtOH	2- propanol
Hexane	Toluene

A. Cell Harvest and Lipid Extraction

1. After washing cells twice with DPBS, add 2 ml. DPBS/1mM EDTA and scrape cells loose from dish surface.
2. Transfer the cells to a 15 ml. polypropylene conical tube. Pipette 20 times to suspend cells.
3. Transfer 400 µL to a 1.5 ml. eppendorf tube for DNA assay. To these, add 20 µL 50% EtOH in H_2O. Store at -20˚C until assay.
4. To the remaining 1.6 ml. cells, add 6 ml. $CHCl_3$/MeOH (1:2 *v*:*v*).
5. Add 5 µL of each surrogate: D_7 Cholesterol and D_7 4β-Hydroxycholesterol. Make note of the concentrations of these standards. Vortex well.
6. Centrifuge at 2400 rpm for 5 minutes (eppendorf 5810 R with swinging bucket rotor).
7. Decant supernatant into a fresh 15 ml. polypropylene conical tube. Discard pellet.
8. To the supernatant, add 2 ml. each of $CHCl_3$ and DPBS. Vortex well.
9. Centrifuge at 2400 rpm for 5 minutes.
10. Remove organic (lower) phase to a fresh 4 ml. glass vial with Teflon-lined cap using a 9 inch Pasteur pipette.
11. Dry the organic phase under nitrogen with gentle heating (37˚C).

B. Sterol Purification

1. Dissolve dried lipids in 1 ml. toluene.
2. Assemble 100mg silica SPE columns on a vacuum apparatus. Vacuum should be adjusted so that flow rate is approximately 2 ml. per minute.
3. Condition the column with 2 ml. hexane. Discard eluate.
4. Apply lipids dissolved in toluene. Discard eluate.
5. Wash the column with 1 ml. hexane. Discard eluate.
6. Elute cholesterol and oxysterols with 8 ml. of 30% 2-propanol in hexane. Collect eluate in glass tubes.
7. Transfer eluate to 8 ml. glass vial. Dry under nitrogen with gentle heating (37˚C).
8. Dissolve dried sterols in 400-500µL 10% H_2O in MeOH.
9. Add d_{7-} 7oxocholesterol

Storage: DNA samples are stored at -20°C until analysis. Sterol samples are stored at 4°C

II. Positive ESI Liquid Chromatography Mass Spectrometry (ESI- LC/MS)

The LC/MS protocol outlined below is for the analysis of sterols in purified cell extracts (part I). Sterols were resolved by reverse-phase HPLC using a binary solvent system and gradient elution was performed on a C18 RP-HPLC column. The HPLC was coupled to a triple quadrupole MS with an ESI source. The MS was operated in multiple reaction monitoring (MRM) mode with transitions optimized for each sterol of interest. Sterols were quantified using the internal standards, surrogate, and relative response factor (RRF) of each sterol of interest.

A. Solutions:

1. Mobile Phase A
   Methanol w/ 5mM ammonium acetate

2. Mobile Phase B
   High Purity water w/ 5mM ammonium acetate

Mobile phases A and B were sparged with Helium for 5 minutes.

3. Surrogates

Two deuterated surrogates are added to cells before extraction:

Compound	**Source**
Cholesterol (D_7) in MeOH	Cambridge Isotope Laboratories
4β- Hydroxycholesterol (D_7) in $CHCl_3$	Avanti Polar Lipids

4. Internal Standard

7 Ketocholesterol (D_7) from Avanti Polar Lipids

B. Compounds of interest

We are monitoring the following compounds via Selected Reaction Monitoring

Compound	**MRM Pair**
22r-Hydroxycholesterol	420/385
24-Hydroxycholesterol	420/385
25-Hydroxycholesterol	420/367
26-Hydroxycholesterol	420/385
24,25-Epoxycholesterol	418/383
7α-Hydroxycholesterol	385/367
7-Ketocholesterol	401/383
5/6β Epoxycholesterol	420/385
5/6α Epoxycholesterol	420/385
4β-Hydroxycholesterol	420/385
Zymosterol	385/367
Desmosterol	402/367
7-Dehydrocholesterol	385/367
3keto cholestene	385/367
Lathosterol	404/369
Cholesterol	404/369
Lanosterol	444/409
Cholestanol	404/387
24-Dihydrolanosterol	429/411
3,16dioxo cholestenoic acid	429/411
TriOH cholesterol	401/383
4-chol-27acid-3one	415/397
4-chol-22OH-3one	401/383
4-chol-24OH-3one	401/383
4-chol-25OH-3one	401/383
4-chol-2OH-3one	401/383
20-Hydroxycholesterol	385/367
4-chol-26(25r)OH-3one	401/383
4-chol-26(25s)OH-3one	401/383
3keto,26cholestene	401/383
8(14) cholesten 3β,15α diol	385/367
3β,15α cholestanol	422/369
8(14) cholesten 3βOH 15one	401/383
cholestan 3oh 15one	403/385
7α hydroxycholestenone	401/383
8(14) cholesten 3β,15β diol	385/367

3β,15β cholestanol	422/369
7ketocholestanone	401/383
dihydroxyketocholesterol	401/383
19-Hydroxycholesterol	420/385
4,6 Chlestadiene -3-one	383/365
Lathosterone	385/367
5-chol-3-one	385/367
cycloartenol	444/409
Bsitosterol	432/397
Bsitosterone	413/413
3,16dioxo cholestenoic acid	429/411
TriOH cholesterol	401/383
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Lathosterone	385/367
5-chol-3-one	385/367
cycloartenol	444/409
Bsitosterol	432/397
Bsitosterone	413/413
7β-Oxocholesterol (D7)	408/390
7β-hydroxycholesterol (D7)	391/373
4β-hydroxycholesterol (D7)	426/391
7α-hydroxycholesterol (D7)	391/373
25-hydroxycholesterol (D3)	423/370

C. Instrumentation

1. Column Information

Company: Phenomenex
S/N: 336529-4
Packing: Reverse Phase C18
Particle Size: 3μ
Diameter: 2mm
Length: 150mm

This column is maintained at 25°C.

2. HPLC conditions

Total Flow: 0.28ml/min
Gradient: Increased from 70% A to 100% A in 8 min; was maintained at 100% A for 7 min; and then re-equilibrated to the starting conditions for 5 min.

3. API 4000 Q Trap Conditions

CUR: 10.00
CAD: Medium
IS: 5500.00
GS1: 50.00
GS2: 0.00
DP: Variable Depending on MRM pair (45.00-120.00)
EP: 10.00
CE: Variable Depending on MRM pair (10.00-65.00)
CXP: 10.00