Extraction and Analysis of Sterols in Biological Matrices by High Performance Liquid Chromatography Electrospray Ionization Mass Spectrometry

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METHOD

Supplies and Reagents

Phosphate Buffered Saline (PBS)Cell lifters
Glass culture tubes with Teflon™-lined caps (7-, 11-, 14-, 18-ml)
Wheaton Science Products
7-ml 358640
11-ml 358646
14-ml 358647
18-ml 358648
10-ml glass pipettes
Pasteur pipettes
Mechanical pipette pump
Glass vials with Teflon™-lined caps (2-, 4-, 8-ml)
Wheaton Science Products
2-ml W224581
4-ml W224582
8-ml W224584
Solid-phase extraction (SPE) cartridges (100-mg silica, Isolute)
Vacuum apparatus for SPE
0.5% (v/v) isopropanol in hexane
30% (v/v) isopropanol in hexane
HPLC-grade methanol, ethanol, chloroform, hexane, and H_2O
Potassium hydroxide (10 N)
Ammonium acetate
Saponification solution
Hyrolysis solution (6 ml of 10 N KOH diluted to 100 ml with ethanol)
Bligh/Dyer solvent (1:2 [v/v] chloroform:methanol)
Primary and deuterated sterol standards
Luna C_{18} reverse-phase HPLC column (250 × 2 mm; 3 µm-particle size)

Extraction of Lipids from Cultured Cells and Tissues

Extraction of cultured cells begins by placing the culture dishes (6-cm dishes seeded with $2e^6$ cells) on ice. The medium is removed with a glass pipette to a screw-cap glass culture tube and stored for extraction later. Cells are gently washed twice with 3 ml of cold PBS, which is removed and discarded. An additional 1.6 ml of cold PBS are added to the cells, which are then gently scraped from the surface of the dish using a cell lifter. Cells are transferred to a 14-ml screw-cap glass culture tube.
Depending on the desired information, aliquots of the medium or cells may be taken at this stage for assays to determine levels of cytokines, DNA, or protein. Lipids are extracted from the cells using a Bligh/Dyer procedure in which 6 ml of chloroform:methanol (1:2 v/v) is added to the resuspended cells in 14-ml screw-cap glass culture tubes. At this point, deuterated surrogate sterol standards are added for quantitative analysis, as indicated in **Table 1** (see “Quantitation” section for definition of surrogate standards).
Samples are vortexed at high speed for 10 s, then centrifuged at 2600 rpm (1360 rcf) for 5 min to pellet insoluble material. The supernatant is decanted to a fresh 14-ml glass culture tubes and 2 ml each of chloroform and PBS is added. Samples are again vortexed for 10 s and centrifuged at room temperature for 5 min at 2600 rpm. Two liquid phases should now be observed. The organic lower phase is removed using a Pasteur pipette and transferred

to a 4-ml glass vial with a Teflon-lined cap. The upper aqueous phase is discarded. We recommend the use of a mechanical pipette pump (e.g., Scienceware Pipette Pump) versus a pipette bulb for better control when removing liquid near the aqueous–organic interface.

Table 1. Deuterated and primary sterols and the masses and volumes used for quantitative analysis

Compound[a]	**Parts per million (PPM)**
Surrogate mix 1 (10 µl)	
25-Hydroxycholesterol (D3)	4
27-Hydroxycholesterol-(25*R*) (D7)	4
24,25-Epoxycholesterol (D6) (*R*+*S*)	3
7α-Hydroxycholesterol (D7)	4
7-Ketocholesterol (D7)	4
4β-Hydroxycholesterol (D7)	2
Surrogate mix 2 (10 µl)	
Cholesterol (D7)	150
Desmosterol (D6)	80
Internal standard (10 µl)	
6α-Hydroxycholestanol (D7)	4
Sterol mix (10 µl)	
22*R*-Hydroxycholesterol	4
25-Hydroxycholesterol	4
27-Hydroxycholesterol-(25*R*)	4
24,25-Epoxycholesterol	3
7α-Hydroxycholesterol	4
7-Ketocholesterol	4
4β-Hydroxycholesterol	2
Desmosterol	80
7-Dehydrocholesterol	100
Cholestenone	15
Cholesterol	150
Lanosterol	100

a

See **Table 3** for systematic sterol names and **Table 4** for deuterium positions.

The organic phase is evaporated under a gentle stream of dry nitrogen while heating at approximately 35° to counter the evaporative cooling effect. The dried extracts are reconstituted in approximately 200 µl of 95% (v/v) methanol, shaken gently, and transferred to an auto-sampler vial containing the appropriate deuterated internal standard based on the amount given in **Table 1** (see “Quantitation” section for definition of internal standard). An additional 200 µl of 95% methanol is added, the sample is gently shaken, and the solution is transferred to the same auto-sampler vial. This additional rinse ensures maximum transfer of sterols from the sample vial to the

auto-sampler vial. Vortexing or sonicating the sample should not be done, as these treatments can disperse any residual insoluble material into the solution, which in turn adversely affects subsequent HPLC or MS analyses of the extracts. Occasionally, even gently shaking the extract can cause resuspension of insoluble material. If insoluble material is inadvertently resuspended, centrifugation and decanting the supernatant are usually sufficient to clarify the extract. If the final extract is not colourless and free of solid material, then the Bligh/Dyer extraction should be repeated. The auto-sampler vial should be equipped with an appropriate volume-reducing insert if the final volume is less than 500 μl.

This extraction method is also suitable for animal tissues (brain, liver, etc.), plasma, and medium from tissue culture. Adjustments to the volumes of extraction solvents must be made to maintain the water:chloroform:methanol ratio of 1:2:0.8 for the azeotrope and a 2:2:1.8 ratio for formation of the two-phase system. Exact volumes must be determined empirically for each sample type; however, we have found the following information useful as a guide: when extracting lipids from liquid solutions, the entire volume should be treated as the aqueous component; for tissues, the endogenous water content will contribute to the aqueous component in the azeotrope, and different tissues have different water contents. For example, an adult mouse brain (~400 mg) will typically contribute 0.4 ml of water, meaning that 1.2 ml of PBS would be added instead of the 1.6 ml suggested above. Final volumes must also be adjusted depending on initial mass of the sample and lipid content. Tissues often require homogenization in PBS using a Potter-Elvehjem device or a polytron prior to addition of chloroform and methanol, and again after addition of these organic solvents. The deuterated sterol standards surrogates should be added after homogenization to avoid nonspecific adsorption.

Saponification of Lipid Extracts

Saponifying steryl esters to free sterols requires additional steps in the extraction and purification scheme but is a more judicious use of time and resources compared to employing two HPLC-ESI-MS methods, which can more than double instrument time.

Hydrolysis reagents should be prepared immediately prior to use according to the prescribed method already listed. The lipid extract is dried under nitrogen at approximately 35° in a glass vial with a Teflon-lined cap, and 1 ml of hydrolysis solution is added. The vial is capped, vortexed for 10 s at maximum speed, heated to 90° for 2 h, and then allowed to cool to room temperature. Lipids are extracted from the solution using a modified Bligh/Dyer extraction in which ethanol is substituted for methanol as follows. The hydrolysed lipid solution is transferred to a 14-ml screw-cap glass culture tube using a Pasteur pipette, and the glass vial is rinsed once with 1 ml of ethanol. To the 2-ml volume of hydrolysed extract in ethanol, 2 ml of chloroform and 1.8 ml of PBS are added. Samples are vortexed for 10 s at maximum speed and centrifuged at 2600 rpm for 5 min. Two phases should be observed, and the organic phase (lower) is removed using a Pasteur pipette and placed in a fresh 4-ml glass vial with a Teflon-lined cap, dried down, and reconstituted in 95% methanol.

Solid-Phase Extraction

Bulk-lipid extracts made with the Bligh/Dyer procedure contain many nonpolar species such as steryl esters, monoacylglycerols, diacylglycerols, and triacylglycerols, which are all strongly retained on reverse phase HPLC columns. Accumulation of these compounds on the column can lead to changes in retention time, decreased resolution, and increased back pressure. For these reasons, it is often advisable to resolve the major lipid classes by SPE prior to HPLC-MS analysis. Sterols can be isolated from other classes of lipids by sequential development of a single SPE column and, with the appropriate solvents, oxysterols, or sterols containing additional hydroxy-, oxo-, or epoxy-groups, can be separated from cholesterol and other sterols that contain a single hydroxyl functional group.

For routine SPE a 100-mg Isolute silica cartridge (Biotage, Charlottesville, VA) is prewashed by passing 2 ml of hexane through the column. A dried lipid extract prepared as previously described (with or without saponification) is dissolved in 1 ml of toluene and passed through the cartridge. Nonpolar compounds such as cholesteryl esters are eluted first with 1 ml of hexane. Cholesterol and other related sterols are eluted next with 8 ml of 30% isopropanol in hexane. The eluted sterols are dried down and then resuspended in 95% methanol prior to analysis by HPLC-MS.

To resolve sterols containing a single hydroxyl group from those containing more than one hydroxyl group (e.g., cholesterol from oxysterols), the SPE column is pre-washed and loaded with lipids from the extraction procedure as described above. Steryl esters are again eluted from the column with 1 ml of hexane. Cholesterol and other mono-hydroxy sterols are then eluted with 8 ml of 0.5% isopropanol in hexane. Oxysterols are eluted next with 5 ml of 30% isopropanol in hexane. Eluates from the individual chromatographic steps are dried under nitrogen and resuspended in 95% (v/v) methanol/water. Our laboratory has determined that, in general, a 400-μl final volume for 5 to 10 × 10^6 cells is suitable for the instrumental analysis described in the "Analysis by HPLC-ESI-MS" section. The final volume-to-cell number ratio can be scaled to accommodate varying amounts of cells.

Analysis by HPLC-ESI-MS

High performance liquid chromatography

Sterols are resolved using reverse-phase HPLC (RP-HPLC). A 10-µl aliquot of lipid extract (in 95% methanol) is loaded onto a RP-HPLC column (Luna C_{18} 2 × 250 mm, 3-µm particle; Phenomenex, Torrance, CA) equipped with a guard column (C_{18}, 4 × 2 mm). From this point on, lipids are resolved by gradient elution. The gradient program begins with 100% Solvent B (85% methanol, 5 m*M* ammonium acetate) for 2 min, and is thereafter ramped to Solvent A (100% methanol, 5 m*M* ammonium acetate) over a 13-min period and held for 10 min. Solvent B is then passed through the column for 5 min to re-equilibrate the column prior to the next run. The flow rate is 0.25 ml/min, and the column is maintained at 30° using a column oven.

Electrospray ionization mass spectrometry

The HPLC is coupled to a 4000 QTrap triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) through a Turbo V™ ESI source. The source is operated in the positive (+) mode with a spray voltage of 5500 V, a curtain gas of 15 psi (nitrogen), and ion Source Gas 1 and 2 set to 60 and 20 psi, respectively (both nitrogen). Gas 2 is maintained at 50°. The mass spectrometer is operated in standard reaction monitoring (SRM) mode to achieve maximum selectivity. To increase signal intensity, the first quadrupole is set to low resolution and the third quadrupole is set to unit resolution. These settings allow ions of wider mass distribution to pass through quadrupole 1 into the collision cell where ions are fragmented and resolved by quadrupole 3. Individual transitions, optimal declustering potentials, collision energies, limits of detection, and linear ranges are given in **Table 2**. SRM pairs are chosen by infusing individual sterols into the MS and generating product ion MS/MS from which these values are selected and optimized. Examples of product-ion spectra from several different types of sterols are shown in **Fig. 1**. A more comprehensive list of sterol product-ion MS can be found at http://www.lipidmaps.org. Common ions formed for most sterols by electrospray include $[M+NH_4]^+$, $[M+H]^+$, $[M+H–H_20]^+$, and $[M+H–2H_2O]^+$. Note that the $[M+H]^+$ ion does not result from gaining a proton during electrospray, but is likely the result of losing gaseous ammonia (NH_3) from the NH_4 adduct.

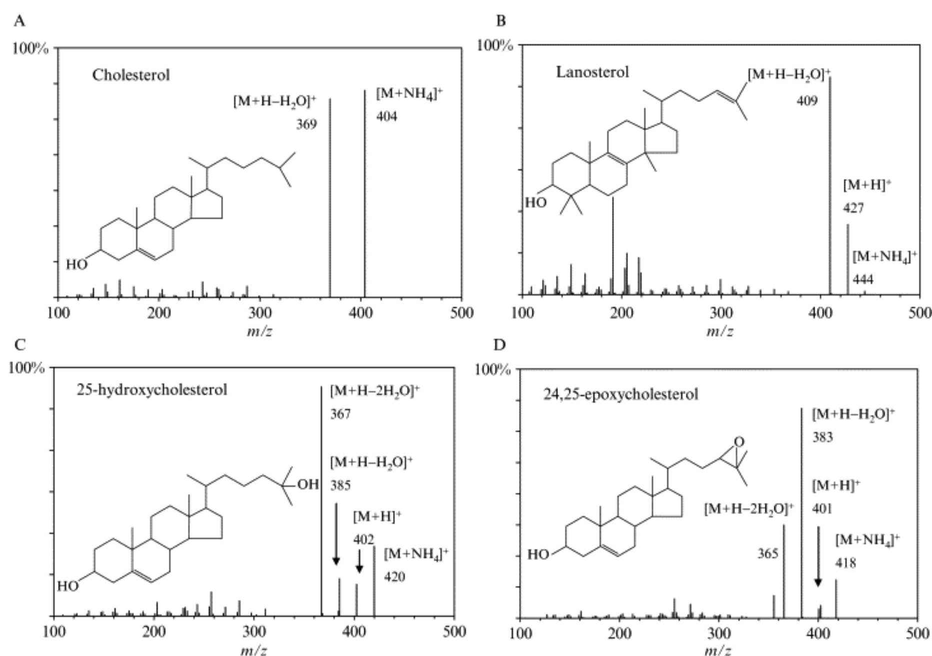

Figure 1. Product-ion mass spectra for selected sterols. See http://www.lipidmaps.org for additional spectra.

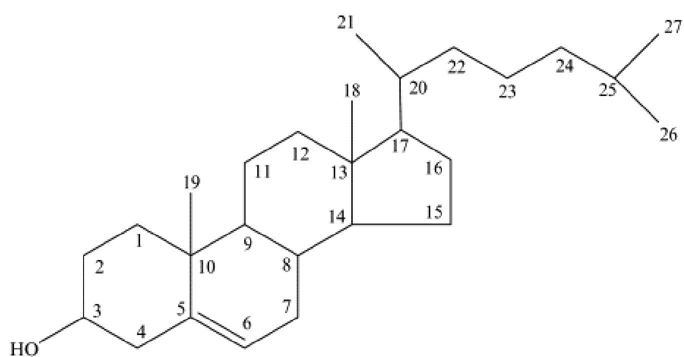

Figure 2. Structure of cholesterol with positional numbering system.

Table 2. Molecular weight, instrumental parameters, and figures of merit for representative sterols

Common name	Peak # (Fig. 3)	MW[a]	SRM ions	DP (v)	CE (v)	Dwell time (msec)	RT (min)	LOD (fmol on-column)	Linear range (fmol on-column)
22*R*-Hydroxycholesterol	1	402.7	420/385	50	15	110	10.5	50	5.00e1–1.25e5
24-Hydroxycholesterol (*R*+*S*)	2/4	402.7	420/385	50	15	110	12.2	60	6.50e1–1.50e5
25-Hydroxycholesterol	3	402.7	420/367	50	15	110	13.3	25	2.50e1–2.50e5
27-Hydroxycholesterol-(25*R*)	5	402.7	420/385	50	15	110	13.9	55	5.50e1–1.25e5
24,25-Epoxycholesterol	6	400.4	418/383	45	12	80	14.5	20	2.00e1–1.25e5
7α-Hydroxycholesterol	7	402.7	385/367	50	15	110	16.5	60	6.00e1–1.25e5
7-Ketocholesterol	8	400.6	401/383	80	35	70	17.3	85	9.00e1–6.30e4
5,6β-Epoxycholesterol	9	402.7	420/385	50	15	110	18.7	5	5.00e0–8.70e4
5,6α-Epoxycholesterol	10	402.7	420/385	50	15	110	19.5	5	6.00e0–8.70e4
4β-Hydroxycholesterol	11	402.7	420/385	50	15	110	20.2	25	2.50e1–6.20e4
Zymosterol	*a*	384.6	385/367	85	20	70	N/A	N/A	N/A
Desmosterol	12	384.6	402/367	55	20	110	21.9	2000	6.50e4–3.20e6
7-Dehydrocholesterol	13	384.6	385/367	85	20	70	22.6	520	5.20e2–3.30e6
Cholest-4-en-3-one	14	384.6	385/367	85	20	70	23.4	390	3.90e2–1.60e6
Lathosterol	15	386.7	404/369	110	13	70	24	1300	9.00e3–2.50e6

Common name	Peak # (Fig. 3)	MW[a]	SRM ions	DP (v)	CE (v)	Dwell time (msec)	RT (min)	LOD (fmol on-column)	Linear range (fmol on-column)
Cholesterol	16	386.7	404/369	110	13	70	24.3	1000	2.60e4–3.20e6
Lanosterol	17	426.7	444/409	80	15	100	24.9	175	2.30e3–3.00e6
Cholestanol	18	388.7	404/369	110	13	70		*b*	*b*
24-Dihydrolanosterol	19	428.7	429/411	100	17	70		*b*	*b*

Key to abbreviations: CE, collision energy; DP, declustering potential; LOD, limit of detection; MW, molecular weight; N/A, not applicable; RT, retention time; SRM, selected reaction monitoring.

[a] Compound no longer commercially available. Existing material is limited and impure; thus, LOD and linear ranges are not measured.

[b] Standards are of questionable purity; thus, LOD and linear range were not measured.

QUANTITATION

Deuterated analogues of sterols are used for quantitation because they have similar physical properties as non–isotopically labelled (i.e., endogenous) sterols, but can be resolved from the latter by MS due to their increased mass. Quantitation relies on the principle of isotope dilution MS in which known amounts of deuterated sterol standards are added to the biological sample at the beginning of the extraction and are thereafter carried through the extraction procedure with endogenous sterols. The amount of endogenous sterol in the cell or tissue extract is then calculated using a predetermined relative response factor (RRF) between peak areas and masses of the deuterated versus endogenous sterols. To obtain RRFs, a standard mixture of deuterated surrogates and internal standard is prepared as indicated in **Table 1** prior to instrumental analysis. An autosampler vial is fitted with a 500-µl insert, and ~400 µl of 95% methanol is added. Deuterated surrogates and internal standards are then added to the solution in the exact manner in which they were added to the sample as previously described (same volume, pipette, stock mixtures, etc.). Corresponding primary sterols are added to the mixture as well. The masses and volumes for all compounds are given in **Table 1**.
The quantitation mixture is analysed at the beginning and end of the instrument sequence, and after every 8 to 10 samples. Using the masses of standard added to the quantitation mixture and the areas under the elution curves generated by HPLC-ESI-MS, a response factor can be generated using Equation 1.

$$(1)\quad RRF = (Area_{Analyte} \times Mass_{std}) / (Area_{std} \times Mass_{Analyte})$$

The response factors are averaged over the course of the sequence to account for any instrumental drift. Should the response factor drift excessively between two standard analyses, specific response factors should be used only for the samples they bracket. This approach establishes the relationship between mass and peak area, which can be used to quantitate the amount of each sterol present in individual samples. Equation 2 takes into account the area under the elution curve for the sterol of interest ($Area_{Analyte}$), the deuterated analogue ($Area_{std}$), the mass of the deuterated analogue ($Mass_{std}$) added to the sample, and the RRF generated with Equation 1.

$$(2)\quad Mass_{Analyte} = (Area_{Analyte} \times Massstd)/(Area_{std} \times RRF)$$

Equation 2 can be seen as a rearrangement of Equation 1 in which the RRF established for each sterol and deuterated analogue in Table 6.1 is incorporated.
Deuterated analogues are not available for all sterols. To overcome this problem, an alternate deuterated analogue of similar chemical composition to that of the analyte to be measured can be substituted. For example, quantitative analysis of 22-hydroxycholesterol can be performed using (d3) 25-hydroxycholesterol as an alternative surrogate. Common primary and deuterated sterol standards and their sources are given in **Table 3, Table 4.**

Table 3. Sources of representative sterol standards

Systematic name[a]	**Common name**[d]	**MW**	**Source**
Cholest-5-en-3β,22-diol (22*R*)	22*R*-Hydroxycholesterol	402.7	Sigma Aldrich[b]
Cholest-5-en-3β,24-diol	24-Hydroxycholesterol(*R*+*S*)	402.7	Avanti Polar Lipids[c]
Cholest-5-en-3β,25-diol	25-Hydroxycholesterol	402.7	Avanti Polar Lipids
Cholest-(25*R*)-5-en-3β,27-diol	27-Hydroxycholesterol-(25*R*)	402.7	Avanti Polar Lipids
24,25-Epoxycholest-5-en-3β-ol	24,25-Epoxycholesterol	400.6	Avanti Polar Lipids
Cholest-5-en-3β,7α-diol	7α-Hydroxycholesterol	402.7	Avanti Polar Lipids
7-Keto-5-cholesten-3β-ol	7-Ketocholesterol	400.6	Avanti Polar Lipids
5,6β-Epoxy-5β-cholestan-3β-ol	5,6β-Epoxycholesterol	402.7	Avanti Polar Lipids
5,6α-Epoxy-5α-cholestan-3β-ol	5,6α-Epoxycholesterol	402.7	Avanti Polar Lipids
Cholest-5-en-3β,4β-diol	4β-Hydroxycholesterol	402.7	Avanti Polar Lipids
8,24(5α)-Cholestadien-3β-ol	Zymosterol	384.7	*a*
Cholest-5,24-dien-3β-ol	Desmosterol	384.7	Avanti Polar Lipids
Cholesta-5,7-dien-3β-ol	7-Dehydrocholesterol	384.7	Sigma Aldrich
Cholest-4-en-3-one	Cholestenone	384.7	Sigma Aldrich
Cholest-7-en-3β-ol	Lathosterol	386.7	Steraloids[e]
Cholest-5-en-3β-ol	Cholesterol	386.7	Avanti Polar Lipids
Lanosta-8,24-dien-3β-ol	Lanosterol	426.7	Steraloids
Cholestan-3β-ol	Cholestanol	388.7	Sigma Aldrich
5α-Lanost-8-en-3β-ol	24-Dihydrolanosterol	428.7	Steraloids

a Compound no longer commercially available.
b St. Louis, MO.
c Alabaster, AL.
d See www.lipidmaps.org for systematic nomenclature of sterols.
e Newport, RI.

Table 4. Sources of deuterated sterol standards

Common name (deuterium positions)[a]	**MW**	**Source**
24-Hydroxycholesterol(D6) (26,26,26,27,27,27-D6)	408.7	Avanti Polar Lipids[b]
25-Hydroxycholesterol (D3) (26,26,26-D3)	405.7	Avanti Polar Lipids
27-Hydroxycholesterol-(25*R*)(D5) (26,26,26,27,27-D5)	407.7	Medical Isotopes, Inc.[c]
24,25-Epoxycholesterol (D6) (*R*+*S*) (26,26,26,27,27,27-D6)	406.6	Avanti Polar Lipids
6α-Hydroxycholestanol(D7) (25,26,26,26,27,27,27-D7)	411.7	Avanti Polar Lipids
7α-Hydroxycholesterol(D7) (25,26,26,26,27,27,27-D7)	409.7	Avanti Polar Lipids
7β-Hydroxycholesterol(D7) (25,26,26,26,27,27,27-D7)	409.7	Avanti Polar Lipids
7-ketocholesterol(D7) (25,26,26,26,27,27,27-D7)	407.6	Avanti Polar Lipids
5,6α-Epoxycholesterol (D7) (25,26,26,26,27,27,27-D7)	409.7	Avanti Polar Lipids

Common name (deuterium positions)[a]	MW	Source
4β-Hydroxycholesterol(D7) (25,26,26,26,27,27,27-D7)	409.7	Avanti Polar Lipids
Desmosterol (D6) (26,26,26,27,27,27-D6)	390.7	Avanti Polar Lipids
Cholesterol (D7) (25,26,26,26,27,27,27-D7)	393.7	Avanti Polar Lipids

A See **Table 3** for systematic sterol names.
B Alabaster, AL.
C Pelham, NH.

Data

An HPLC-ESI-MS chromatogram of a mixture of sterols is shown in **Fig. 3A.** Using our standard gradient elution program, sterols elute from the column between 10 and 28 min, and, in general, more hydrophilic oxysterols elute before cholesterol and other more hydrophobic sterols. Experience has shown that the earliest eluting sterols (i.e., the most hydrophilic) extracted from cultured mammalian cells are trihydroxy compounds, which elute several minutes before a 22-hydroxycholesterol standard. The latest eluting sterol (i.e., most hydrophobic) is 24-dihydrolanosterol. All other sterols detected in this laboratory elute between these two extremes. Peak widths are generally less than 20 s at the full-width, half maximum setting, which gives excellent resolution. Note that some sterols show multiple SRM pairs for a single compound. For example, 4β-hydroxycholesterol (peak 11**, Fig. 3B**) shows an SRM signal of 420/385, 420/367, and 385/367 u. These additional SRM pairs add confidence to the identification of a specific sterol and can provide further information regarding compound identification based on their relative intensities.

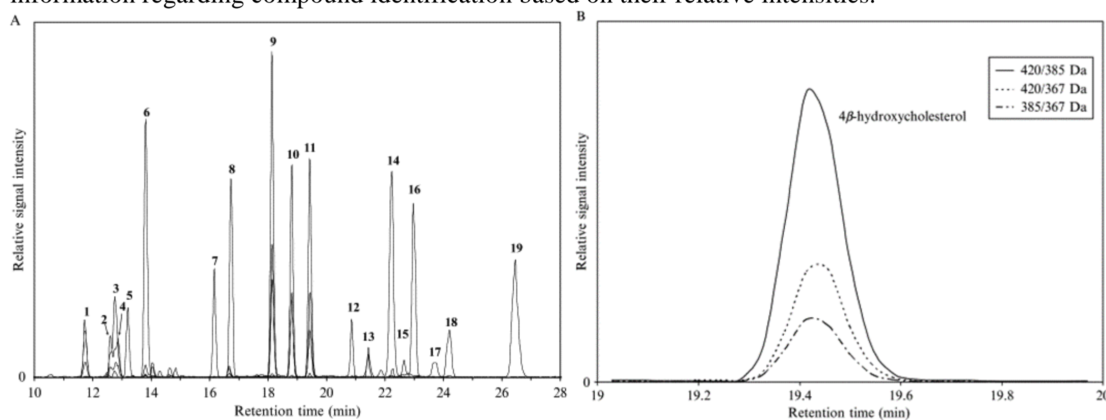

Figure 3. **(A)** Reconstructed SRM ion chromatogram derived from the analysis of a mixture of common sterols. Corresponding peak numbers are given in **Table 2**. The baseline is truncated for clarity to show only 10 to 28 minutes. **(B)** Expanded view of multiple SRM pairs for peak 11 (4β-hydroxycholesterol).

An HPLC-ESI-MS chromatogram derived from analysis of a Bligh/Dyer extract from wild-type mouse brain is shown in **Fig. 4**. Early in the chromatogram, a group of compounds was observed that was not retained by the stationary phase and eluted with the solvent front. This observation is common in biological extracts, especially when no additional pre-purification or separation is performed. Multiple compounds are observed eluting in the sterol region, some of which can be identified based on SRM pairs and retention times. For example, 24-hydroxycholesterol eluting at 12.8 min is abundant in the brain and readily observed in the chromatogram. Other sterols identified in the brain extract include 24,25-epoxycholesterol (13.5 min), 4β-hydroxycholesterol (19.7 min), desmosterol (21.3 min), cholesterol (23.7 min), and lanosterol (24.3 min).
Note that the region around the cholesterol peak is shown at one-tenth scale. The chromatographic peak resulting from cholesterol is typically an order of magnitude or larger than any other sterol, which reflects the large abundance of this sterol in most mammalian tissues and cells. In this example, the cholesterol peak has a split top indicative of poor chromatographic performance due to column overloading. Injecting a smaller volume or a more dilute sample will improve peak shape. Additional sterols are present in the extract as shown by signals from other SRM pairs. These represent known or unidentified sterols, or non-sterol compounds that happen to produce a signal identical to one or more of the SRM pairs but do not match the retention time of any of the sterol standards.

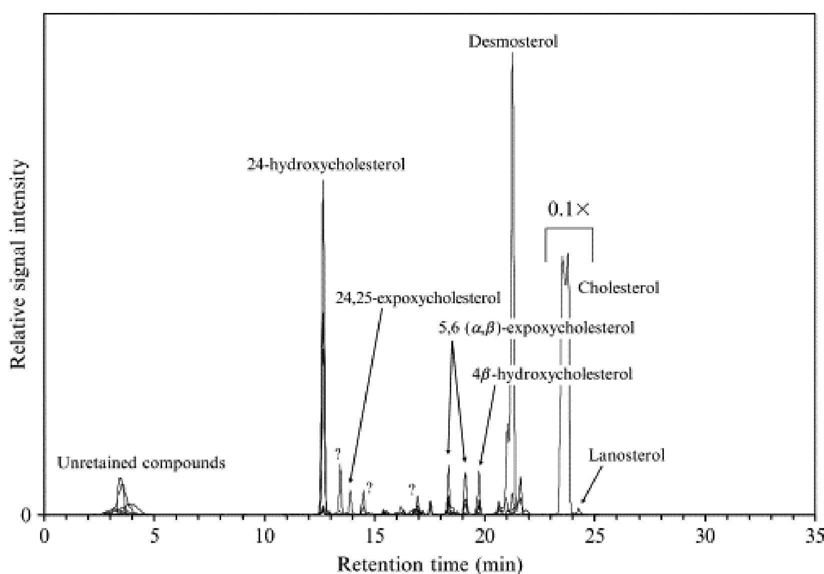

Figure 4. Reconstructed SRM chromatogram of a Bligh/Dyer extract of wild-type mouse brain. Known sterols are identified by name, and unknown compounds with sterol-like signatures are indicated with a "?." Note the reduced scale (×0.1 between 23 and 25 min).

Quantitation

A practical description and discussion of quantitative MS by isotope dilution are provided here. For additional information, see Duncan 2006, United States Environmental Protection Agency (USEPA) 2003. Surrogate standards are deuterated analogues added to the sample prior to extraction and carried through the extraction process with the endogenous compounds. Theoretically, deuterated analogues will behave identically to their non-isotopically labelled and endogenous counterparts during the extraction process, including any losses due to incomplete transfer, oxidation, degradation, or others. When surrogates are used for quantitation, losses are accounted for and corrected automatically. In contrast, internal standards are those added to the sample after extraction but immediately prior to HPLC-ESI-MS analysis. As internal standards are added after the extraction, they cannot account for losses during extraction.

A combination of surrogate and internal standards provides additional information, allowing for the measure of extraction efficiency. The amount of surrogate recovered at the end of an extraction can be calculated providing extraction efficiency using the mass and peak areas of a surrogate and an internal standard. While there is no specified level of recovery necessary for a suitable extraction, a minimum of 50% recovery is our internal target. Values that are consistently lower than 50% recovery indicate the need for evaluation and modification of the extraction procedure. Occasional recovery values below 50% are typically not of concern but should be evaluated on a case-by-case basis. Extraction efficiency is especially important in sterol analysis as many are present in trace quantities within cells and tissues. The extraction procedure previously described for cells typically yields between 70 and 80% average recovery using (d6) 27-hydroxycholesterol as an internal standard. Common primary and deuterated sterol standards and their sources are given in **Table 3, Table 4**.

Isotope dilution has the benefit of alleviating the analyst from having to know the volumes of the extract, including the final volume. Because the calculations are based on ratios between peak area and mass, no knowledge of extract volume is necessary. The calculated value will have the units (mass/extract) that allow the end user to normalize the mass of sterol to a variety of standard measures, including DNA, protein, cell number, or tissue weight.

Availability and purity of primary and deuterated standards

Primary sterol standards are available from several commercial sources. Some standards are prepared synthetically while others are extracted from biological matrices and purified. It has been our experience that the purity of individual sterols varies greatly depending on the specific sterol and the supplier. For example, cholesterol is available from multiple sources as a highly pure compound (>99%), but 24-dihydrolanosterol has limited

availability and typically contains many impurities. Some stereochemically pure compounds are available, such as 22*R*-hydroxycholesterol, 27-hydroxycholesterol-(25*R*), and 24(*S*)-hydroxycholesterol. Others are currently only available as the racemic mixture. Suppliers generally offer limited analytical data to demonstrate purity, which ranges from a basic thin layer chromatographic (TLC) analysis, to an HPLC-UV/vis analysis and nuclear magnetic resonance (NMR) analysis.

Evaluating the purity of primary and deuterated sterol standards can be complex and time-consuming and may be beyond the scope of many research laboratories. As described in the introductory section, sterols are not well suited for analysis in their native state by most modern analytical techniques. Even when detected, the response between two similar sterols can differ by more than an order of magnitude, which makes it difficult to ascertain relative percent purity without having standards for each impurity detected. HPLC-ELSD is currently the most widely used and non-discriminatory technique for evaluation of sterol purity. HPLC-ELSD is optimal for evaluation of standards but, because it does not provide mass information, has limited applicability with complex extracts. Finally, NMR is often considered a definitive technique for identifying purity and structure of organic compounds; however, access to NMR is not always readily available to those studying sterols.

A collection of deuterated sterol standards and an increasing number of primary sterol standards has become available from Avanti Polar Lipids (Alabaster, AL) as part of the LIPID MAPS consortium. The deuterated sterols available at the time of this writing are listed in **Table 4**, and many of the primary sterol standards available are listed in **Table 3**. Each standard from Avanti has been prepared with a full complement of available analytical data, including HPLC-ESI-MS, ESI-MS, HPLC-ELSD, and NMR. This characterization far exceeds the QA/QC data offered by other commercial vendors of sterol standards at this time. Additionally, Avanti performs routine stability testing of stocked compounds and will notify users should the purity of a given standard fail their specified compliance of ±5%.

Resolving related sterols by HPLC

Many sterols are fully resolved by the chromatographic method previously described; however, some, such as 24-hydroxycholesterol (*R*+*S*) and 25-hydroxycholesterol, co-elute. Mass spectrometry can offer an additional level of resolution by differentiating co-eluting compounds by mass but, because many sterols are isobaric, this dimension is of variable worth. 24-Hydroxycholesterol (*R*+*S*) and 25-hydroxycholesterol share three SRM pairs, but they vary in relative intensities between 24- and 25-hydroxycholesterol. 24-Hydroxycholesterol shows 420/385 u as the most abundant transition, whereas 25-hydroxycholesterol shows 420/367 u as the most abundant transition. In some cases, a difference in the tissue-specific distribution of two closely related sterols obviates the need for HPLC separation. In this example, a variety of tissues contain 25-hydroxycholesterol, but 24-hydroxycholesterol is selectively present in the brain.

We have made an extensive effort to develop a single gradient elution method that resolves all closely related sterols present in a typical mammalian cell, and this effort has largely succeeded. To date, our most favourable results allow for a qualitative identification of the known co-eluting sterols; however, resolution is still inadequate for quantitation. Should resolution of two co-eluting sterols be necessary, various isocratic elution methods can be employed. This approach, however, usually produces broader peaks and lower signal. Also, more hydrophobic sterols, such as cholesterol and lanosterol, will have long isocratic elution times that fall outside of optimal chromatographic conditions. GC-MS remains a viable alternative for the quantitation of 24- and 25-hydroxycholesterol.

Acetonitrile and signal intensity

Some methods describing the analysis of sterols by HPLC-MS utilize acetonitrile as a component of the mobile phase. When we incorporated acetonitrile into the gradient mobile phases, a modest to significant decrease in signal intensity was observed for all sterols analysed by LC-ESI-MS. Oxysterols suffered the largest reduction in signal intensity in the presence of acetonitrile, whereas the reduction observed for other mono-hydroxylated sterols was not as severe. Chromatographic parameters were similar in terms of peak-width and retention time. Thus, reductions in signal intensity are not likely explained by changes in chromatographic performance. We have no clear explanation for this phenomenon at this time, but it appears that acetonitrile has a detrimental effect on the electrospray process and adduct formation, which in turn causes decreased signal intensity for oxysterols. Recent research by has described acetonitrile as a reactive gas-phase reagent with atmospheric pressure ionization methods that may be related to the decreased signal intensity observed here. For this reason, and because no increase is observed in chromatographic resolution when using acetonitrile, the methanol/water gradient previously described was implemented.

Auto-oxidation and use of SPE columns

It has been shown that cholesterol is subject to autooxidation, forming oxidized end-products such as 22-, 24-, 25-, and 27-hydroxycholesterol, and 5/6(α/β)-epoxycholesterol. In our experience, the most readily formed oxidation product is 5/6(α/β)-epoxycholesterol, which has been shown to be physiologically irrelevant. Other oxidation end-products are formed in negligible amounts. We have found that extraction of biological samples using the Bligh/Dyer approach above described typically results in minimal oxidation. When further purification steps are conducted on SPE silica columns, the abundance of 5/6(α/β)-epoxycholesterol increases. A pilot study using deuterated compounds to allow quantitation and the full extraction/SPE procedure previously described showed that as much as 1 to 3% of cholesterol can be oxidized to 5/6(α/β)-epoxycholesterol. No other autooxidation products of cholesterol were detected. Addition of the antioxidant butyl hydroxy toluene (BHT) to the sample prior to extraction produced only a modest decrease in oxidation. In most experiments, we do not consider these oxidation products problematic. Minimizing oxidation by using good lab practices is important, but to eliminate oxidation would require processing the samples in an inert environment, which is not logistically possible for most laboratories. It should be noted that 5/6α- and 5/6β-epoxycholesterol are among the most sensitive sterols analysed by this instrumental method **(Table 2),** which can result in large signals being observed in the SRM chromatograms relative to other signals. This imbalance in relative signal strength adds complexity and, possibly, confusion to the interpretation of the data.

Sample clean-up

Depending on sample type and desired information, additional sample processing may be desirable; however, in addition to the increased formation of oxidation products already described, we find that typical clean-up steps such as saponification and SPE require additional time and resources and produce only limited benefit. While we have described here a comprehensive extraction procedure involving extraction, saponification, and solid-phase purification, the last two steps are not always required for successful analysis of sterols. In specific cases, such as plasma, oxysterols are found as free and esterified compounds.

Saponifying the extracts will increase the amount of free oxysterols present in the extract, resulting in stronger signal intensities for these compounds. Otherwise, unless knowledge of free-to-esterified sterol ratios is required or the final extracts are not suitable for analysis after multiple Bligh/Dyer extractions, we do not recommend performing additional separation and purification steps beyond the Bligh/Dyer extraction. Use of a guard column with the primary HPLC column will trap many highly retained compounds and can be replaced with minimal expense relative to the HPLC column.

Should diminished chromatographic performance be observed, back-flushing the column with a strong reverse-phase solvent such as chloroform or isooctane will often remove most retained compounds and extend the life of the column. Alternatively, HPLC columns are consumable items, and the cost of replacing a column with greater frequency is usually smaller than that associated with the increased equipment and time required for additional separation and purification steps. The chromatographic method outlined above adequately separates a majority of sterols in mammalian cells, and SPE cannot itself resolve problems associated with the analysis of co-eluting sterols or diastereomers. Lastly, with every new step in processing comes the possibility of additional sample loss and, although surrogate standards can account for these losses, if the sterol of interest exists at trace levels, excess processing may reduce its signal below the limits of detection. Figure 5 shows various work-flows for the extraction of sterols that can be tailored to individual user's requirements.

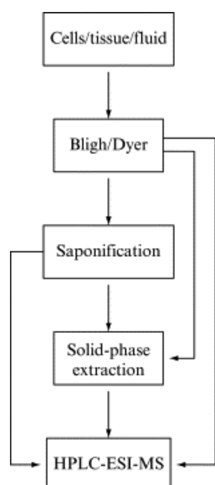

Figure 5. Flow chart of various procedures for the extraction and preparation of sterols from biological extracts.

Residual insoluble material

Regardless of how extensively an extract is separated and purified, residual insoluble material is often present in the final dried extract. When this situation is encountered, we re-dissolve lipids in 95% methanol and make every effort to minimize resuspension of insoluble material. We investigated the composition of this insoluble material by performing additional extractions with both methanol and chloroform followed by ESI-MS analysis. Methanol extraction of particulates showed only small amounts of cholesterol, which may represent residual compound remaining after the extract was transferred to the auto-sampler vial. Chloroform extraction showed no additional sterols, but did dissolve some of the residual material. It would appear that the minor amounts of insoluble material that sometimes form using the above methods is of little concern for subsequent sterol analyses.

Recent experiments (unpublished work) demonstrate that the majority of residual insoluble material previously described is due to the use of plastics in conjunction with organic solvents. We recommend using only glass and Teflon when contact with any organic solvents will occur. The only steps where plastics and organic solvents should come into contact are during the addition of the surrogate and internal standards (when using positive displacement pipettes with disposable tips) or during pre-purification of extracts with SPE columns (the columns are made of plastic). Completely eliminating plastics in these steps would add considerable effort and cost.

Comparing relative peak areas

Individual sterols have varying responses to ESI. The more polar oxysterols (e.g., 24,25-epoxycholesterol) are generally more amenable to ESI ionization compared to less polar compounds (e.g., lanosterol). This difference is reflected in **Table 2**, which shows that limits of detection span several orders of magnitude. Also, later eluting sterols are subject to longitudinal diffusion, which causes peak-broadening and results in a less-intense signal compared to the narrower peaks eluting early in the elution profile. Both of these phenomena prevent interpretation of the relative amounts of sterols in an extract solely by evaluation of their signal intensities. Equivalent signals for two sterols may be the result of very different amounts. Interpretation of relative amounts can only be evaluated by assessing the instrumental response of primary standards for the compounds of interest.

Electrospray using other instrumental platforms

Electrospray ionization and adduct formation of sterols is dependent on the mass spectrometer and, more importantly, the ionization source used. Two laboratories analysing cholesterol by ESI-MS using the previously mentioned parameters but with instruments and/or ionization sources from other manufacturers or other models from the same manufacturer, may obtain different mass spectra or have varying optimal SRM pairs. The method described here is developed and optimized for an Applied Biosystems 4000 QTrap® equipped with a Turbo V™ ionization source. Through discussions with other mass spectrometrists, it is clear that, with some instruments, sterol-ammonium adducts are not observed, but in-source decay products (e.g., loss of NH_3, H_2O, etc.) are. Furthermore, some instruments ionize and transfer sterols to the MS so inefficiently that atmospheric pressure chemical ionization(APCI) is employed in place of ESI. The parameters and results described here are specific to the 4000 QTrap triple quadrupole system, although, on other platforms, they can serve as a foundation from which a suitable HPLC-MS method can be developed, including those for single quadrupole systems.