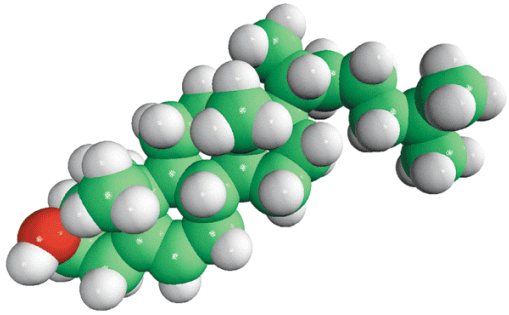
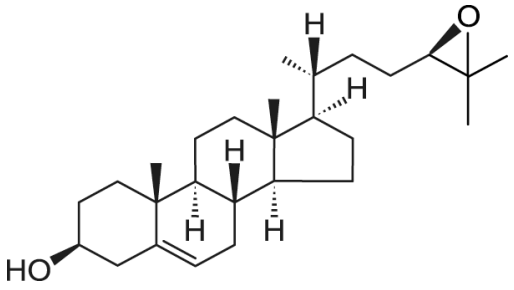
LIPID MAPS Lipidomics Workshop

April 18, 2009

www.lipidmaps.org

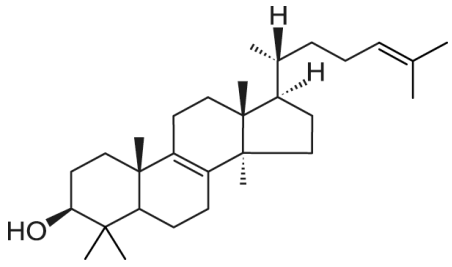


Sterols



Jeff McDonald

Molecular Genetics
The University of Texas Southwestern Medical Center
At Dallas

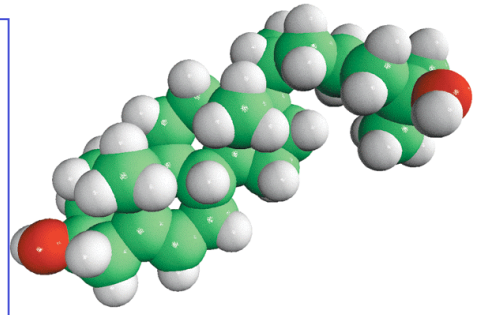


Other LIPID MAPS Sterol Core members:

David Russell

Bonne Thompson, David Bauman, Holly Lincoln

NIH GM-069338



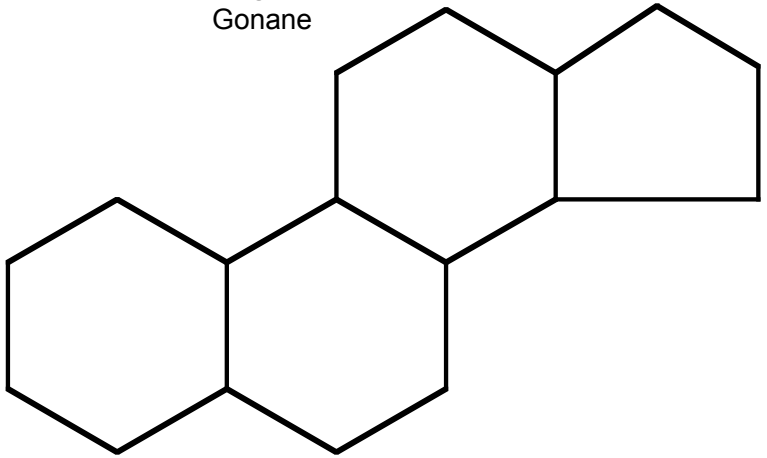
Outline

A. Brief introduction to the lipid class: nomenclature & range of compounds to analyze
B. Sample preparation: solvents, chromatography, recovery,
C. Compound identification: HPLC MS/MS (MRM)
D. Quantitation: Internal standards
E. Alternatives and improvements: New directions with old ideas
G. Remaining challenges and opportunities
H. Discoveries from Sterol analysis in macrophages

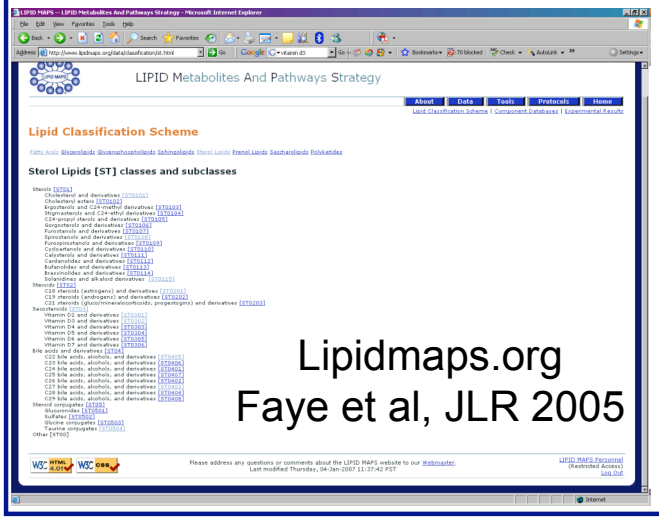
Nomenclature and Range of Compounds to Analyze

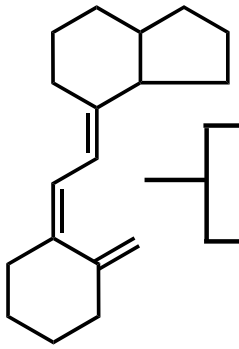
cyclopentanoperhydrophenanthrene

Gonane



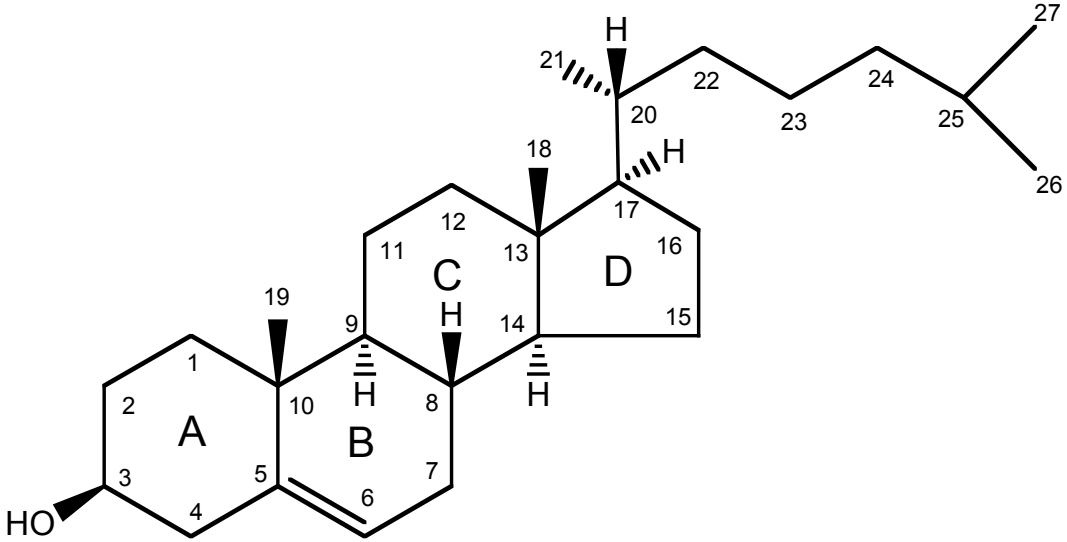
- Sterols → (Cholesterol)
- Steroids → (Testosterone)
- Bile Acids → (Cholic acid)
- Steroid Conjugates → (Cholesterol sulfate)



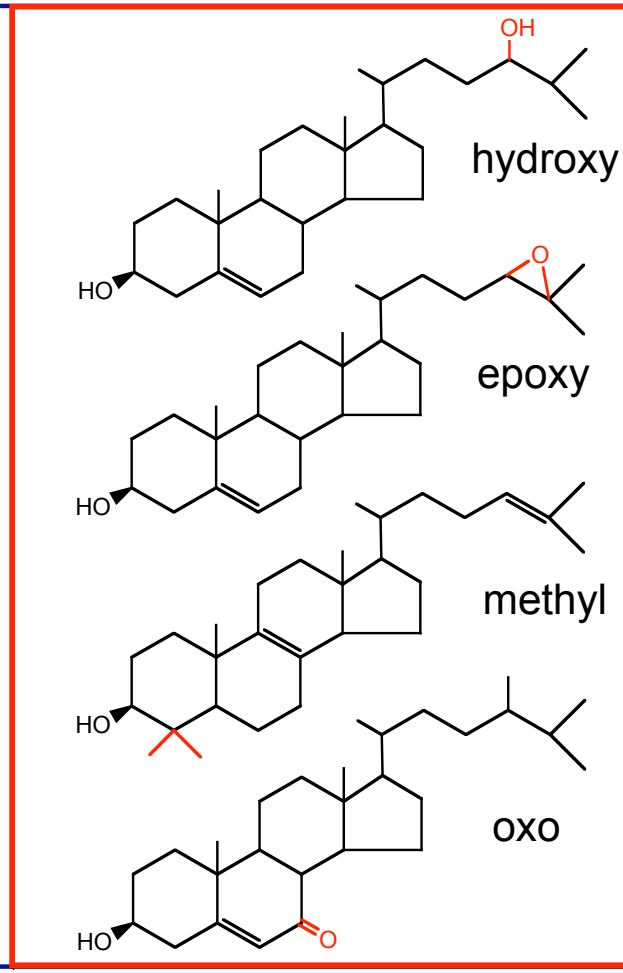


- Secosterols → (Vitamin D3)

Nomenclature and Range of Compounds to Analyze

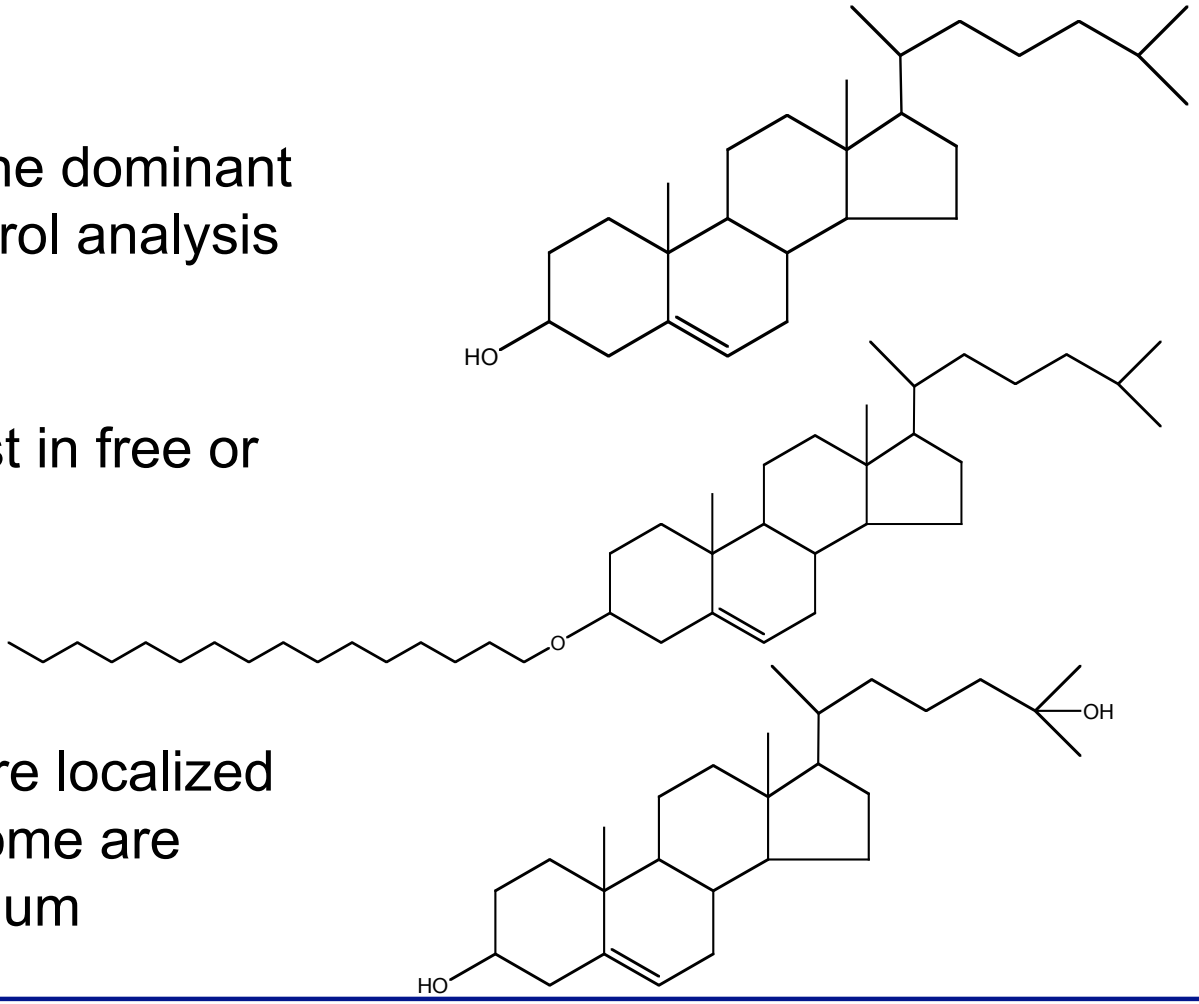


cholest-5-en-3b-ol
Cholesterol

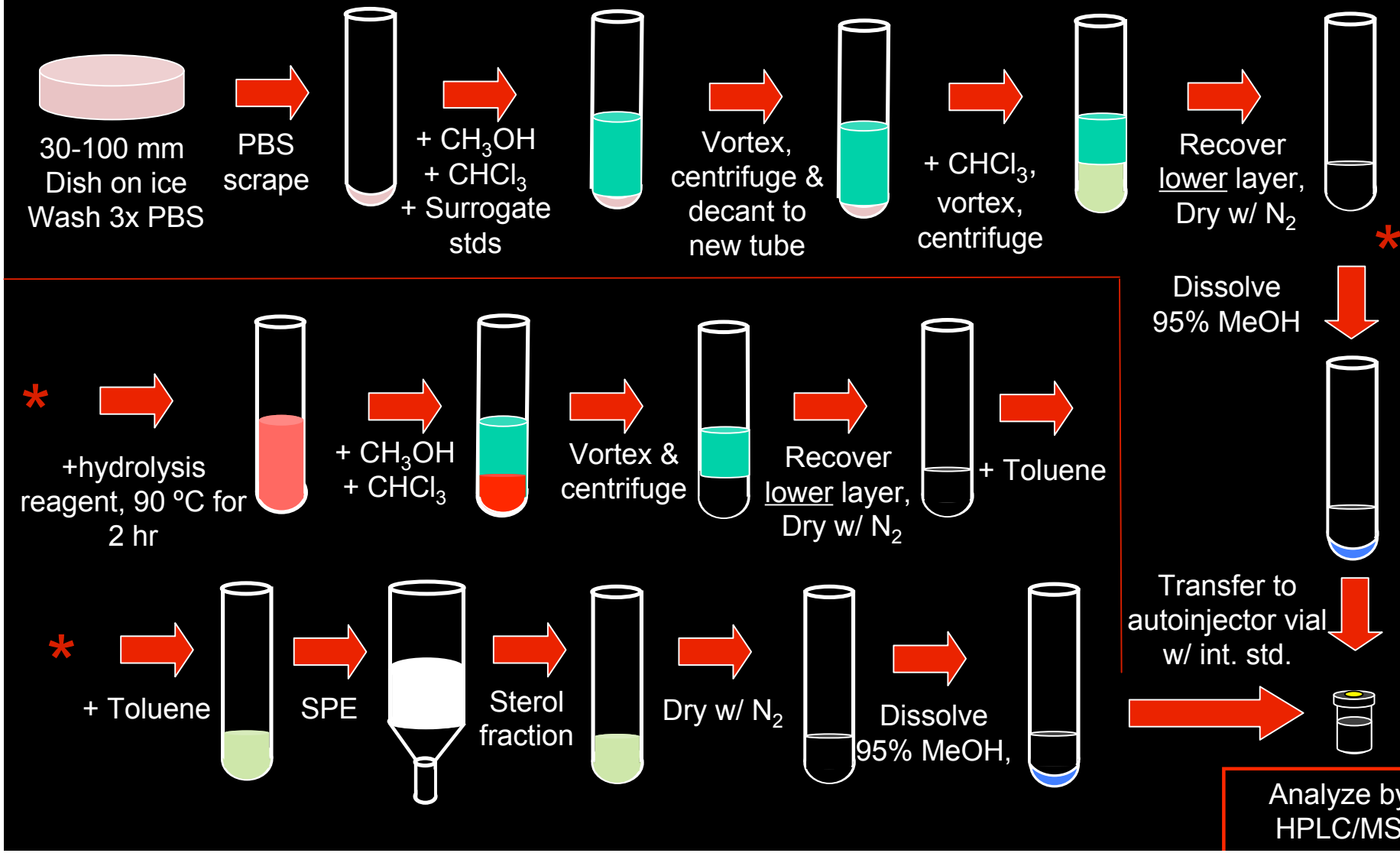


Nomenclature and Range of Compounds to Analyze

- Cholesterol is the dominant species in a sterol analysis
- Sterols can exist in free or esterified forms
- Many sterols are localized in cells only; some are located in medium



Sample Preparation: Extraction of Sterols



Compound identification: HPLC MS/MS; GC/MS

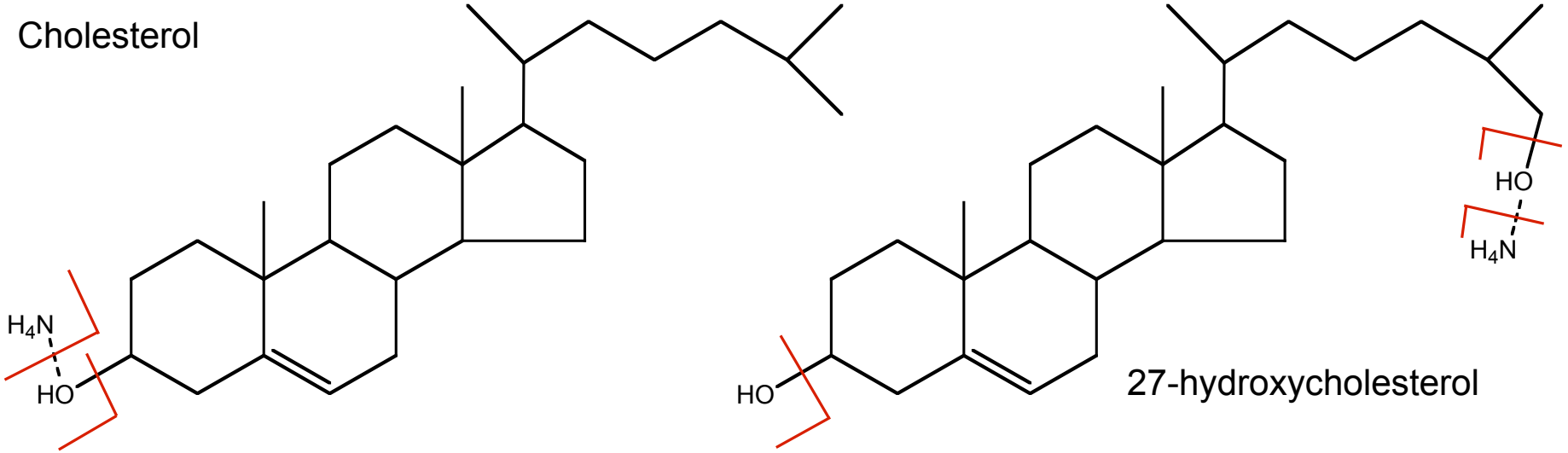
Reverse Phase HPLC

- Phenomenex Luna C_{18}
  - (250 × 2 mm; 3 µm)
- A: 85% MeOH
  B: 100% MeOH
  - Both with 5mM NH_4acetate
- 30 ºC column temp
- 0.25 mL/min, 1 min 100%A to 100%B 15 min, 100%B 10 min, 100%A 5 min

Mass Spectrometry

- Applied Biosystems 4000QTrap
- TurboV Electrospray Ion Source
- Positive (+) mode
- Multiple Reaction Monitoring
- Optimized collision energy, declustering potential

Compound identification: HPLC MS/MS

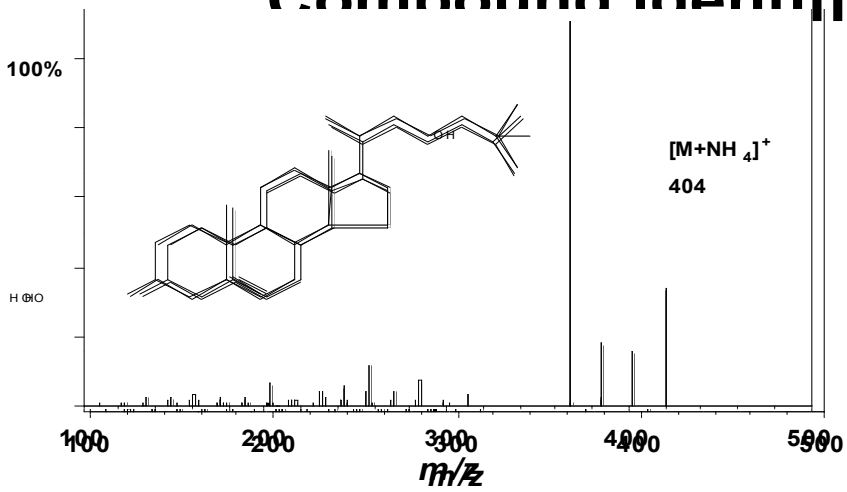


Common Ions Observed by Scan (in-source decay) and Product Ion

$[M+NH_4]^+$ $[M+NH_4\text{-}NH_3]^+$ (M+H) $[M+H\text{-}H_20]^+$ $[M+NH_4\text{-}H_20]^+$?	$[M+NH_4]^+$ $[M+NH_4\text{-}NH_3]^+$ (M+H) $[M+H\text{-}H_20]^+$ $[M+H\text{-}2H_20]^+$ $[M+NH4\text{-}H20]^+$?

No abundant disassociation products beyond loss of waters with ESI

Compound identification: HPLC MS/MS



100%

B. Lanosterol

[M+H -H2O] +
409

[M+H] +
427

[M+NH 4]+
444

100%

C. 25 -hydroxycholesterol

[M+H -2H2O]+
367

[M+H -H2O]+
385

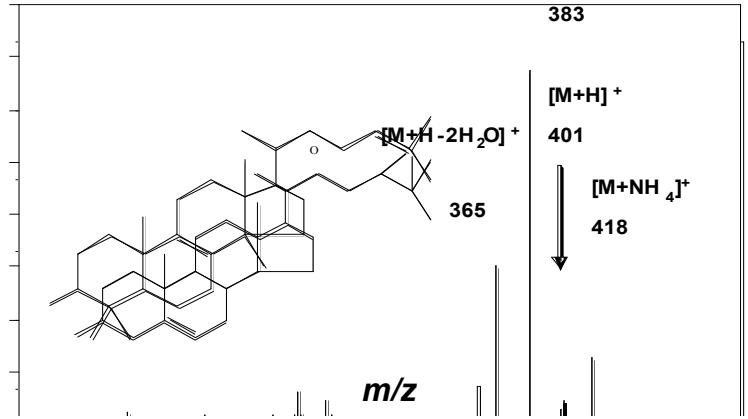
[M+H] +
402

[M+NH 4]+
420

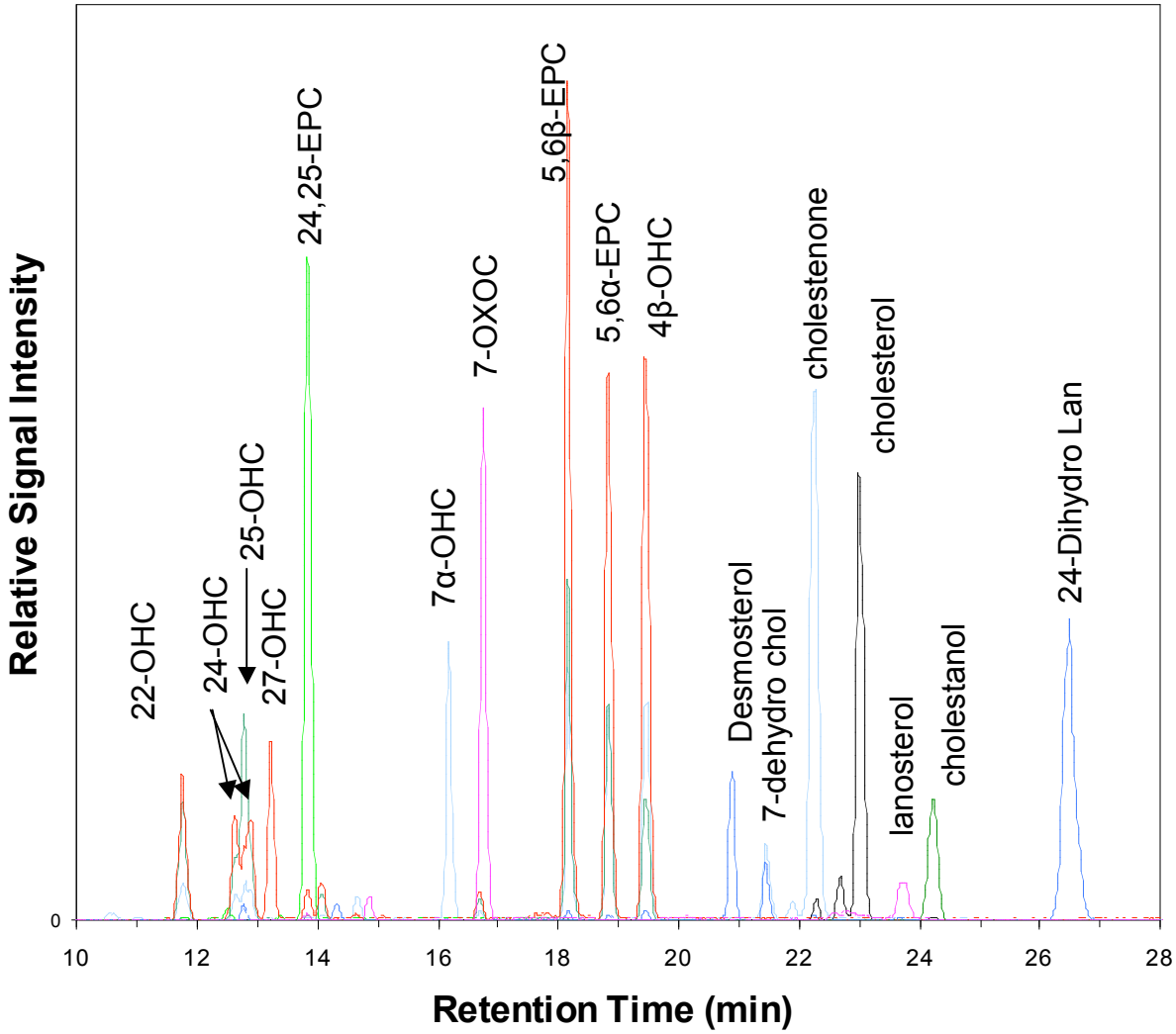
m/z

100%

D. 24,25 -epoxycholesterol



Compound identification: HPLC MS/MS (MRM); GC/MS

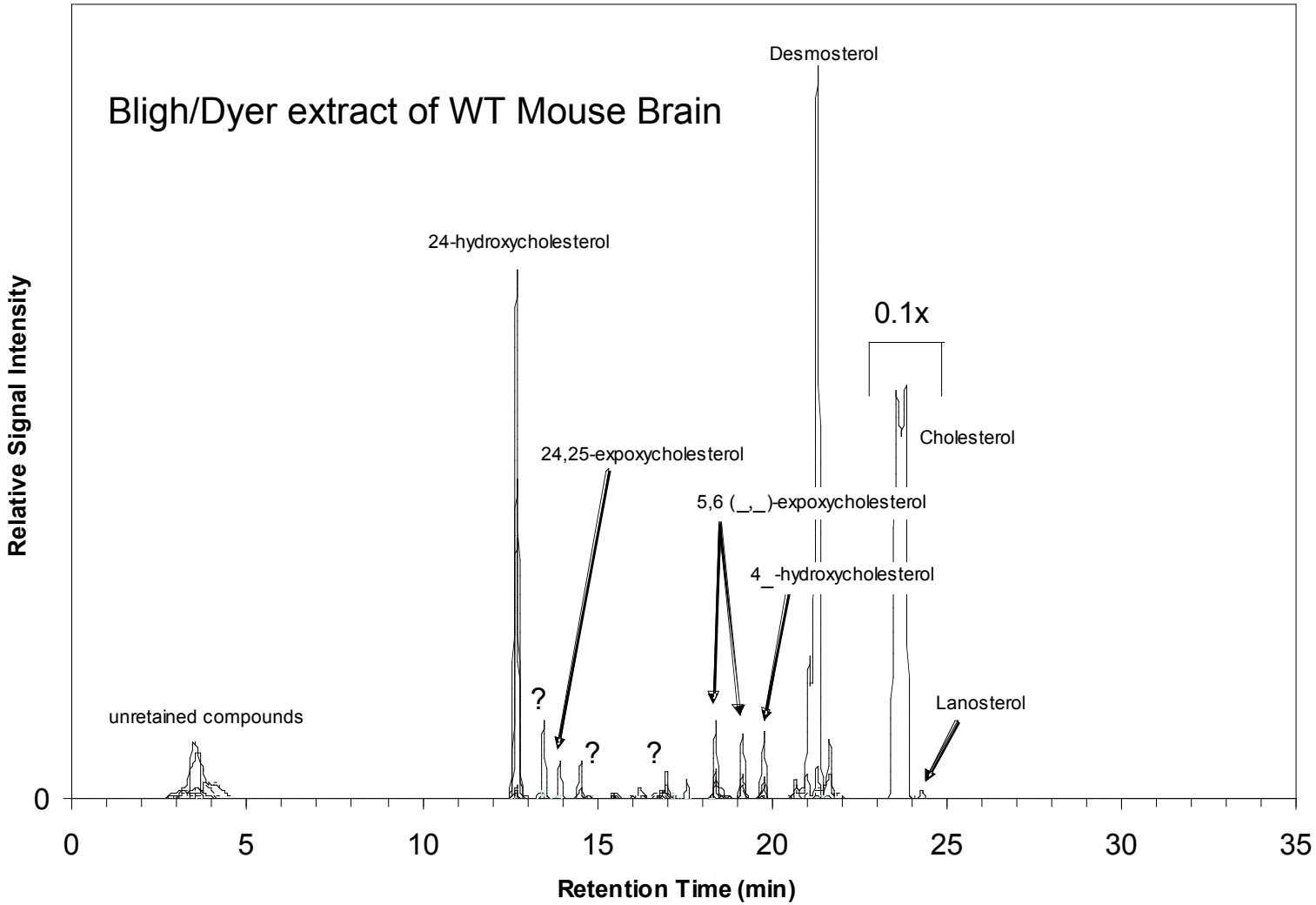


- Multiple Reaction Monitoring
- Most sterols well-resolved
- Peak widths between ~15 and 20 s FWHM

LOD

- 5-100 fmol on-column for oxysterols
- 200-2000 fmol on-column for sterols

Compound identification: HPLC MS/MS (MRM); GC/MS



Quantitation: Internal standards, etc.

COMMON NAME	MW
22*R/S*-hydroxycholesterol	402.7
24-hydroxycholesterol(*R*+*S*)	402.7
25-hydroxycholesterol	402.7
27-hydroxycholesterol-(25*R*)	402.7
24,25-epoxycholesterol	400.6
7α-hydroxycholesterol	402.7
7-ketocholesterol	400.6
5,6α/β-epoxycholesterol	402.7
4β-hydroxycholesterol	402.7
Zymosterol	384.7
Desmosterol	384.7
7-dehydrocholesterol	384.7
Cholestenone	384.7
Lathosterol	386.7
Cholesterol	386.7
Lanosterol	426.7
Cholestanol	388.7
24-dihydrolanosterol	428.7

- Condensed list of sterol standards available from Avanti
- **BOLD** = deuterated analog available
- Avanti Polar Lipid Standards
  - Extensive characterization
    - NMR, LC, MS
    - On-going stability for select compounds
- Additional deuterated and primary standards in progress
- www.avantilipids.com

What Have We Learned in Analyzing Sterols for 5 Years?

A "one-size-fits-all" sterol method has strengths, but also has limitations

Sample Processing

- Cholesterol > 1000x most other sterols (some cases > 20,000x)
  - Requires concentration of final extract; solubility becomes an issue
  - Solubility in MeOH marginal, solubility in MeOH/H_2O poor.

Chromatography

- Coelution of isobaric compounds with HPLC
  - Compound- or class-specific methods

Mass Spectrometry

- Significant spread in signal intensity between similar sterols (ESI)
  - Oxysterols very sensitive, methylated sterols very insensitive
- Matrix interference/ion suppression (ESI)
  - Triglycerides, phospholipids, other abundant lipids
- Native sterols not suitable to precursor ion or neutral loss scanning

Observations on Sample Processing

Base Hydrolysis

- Base hydrolysis necessary for plasma or tissue
  - Generate free sterols from esters
- Eliminates TAGs, phospholipids
- Creates fatty acids, methyl esters
  - Separated by SPE
- Some oxysterols susceptible to high-temperature hydrolysis
  - Room temperature shown to work well
    - Lund et al

Solid-Phase Extraction

- Used to remove remove bulk cholesterol
  - 24,25-Epoxycholesterol, other sterols elute in wrong fraction
- Silica is a reactive substrate
  - Generate oxysterols?
- Separate all oxysterols from all sterols
  - Lund et al Si SPE with hexane/IPA
  - Griffiths et al uses RP C_{18} SPE with EtOH and water
  - NH_2 SPE with hexane/ether showing promise in our laboratory

Observations on Chromatography (HPLC)

- Resolving sterols in single run difficult
  - 24- and 25-OHC; Lathosterol and cholesterol
  - Long run times (≥ 30 min)
- Isocratic elution can be helpful
  - DeBarber et al
- Pentafluorobenzyl stationary phases show unique selectivity
  - Complete separation of 24- and 25-OHC
- CN column with hexane:IPA
  - Excellent separation of all oxysterols
  - 3mm diameter columns (more loading capacity)
  - NP with better reproducibility (water)
  - Fast, hexane as dissolution solvent, low viscosity, compatible with APCI, $
    - Saldanha et al

Observations on Chromatography (GC)

- Superior resolution
  - 200K versus 20K theoretical plates
- Sufficient sensitivity for all non oxysterols
- Library matching with scan mode
- Fast run times with H_2 carrier gas
- Requires derivatization with TMS
- Oxysterols require large samples
  - 1 mL plasma
  - Isolation away from cholesterol, other abundant lipids
- 1-2 uL injections require final sample volume of ~100 uL.
  - Must have clean, isolated sample
- GC-MS (EI) is $70K
  - Short learning curve, low maintenance, implement in almost any lab

Observations on Mass Spectrometry (APCI)

- APCI offers reduced matrix effects/ion suppression
- More uniform response to all sterols
  - Increased detectability of non oxysterols
- Mass sensitive ionization technique
  - ESI is concentration sensitive
- More sample, more signal
- Larger diameter columns, larger injections, reduced sample prep
- Compatible with NP solvents

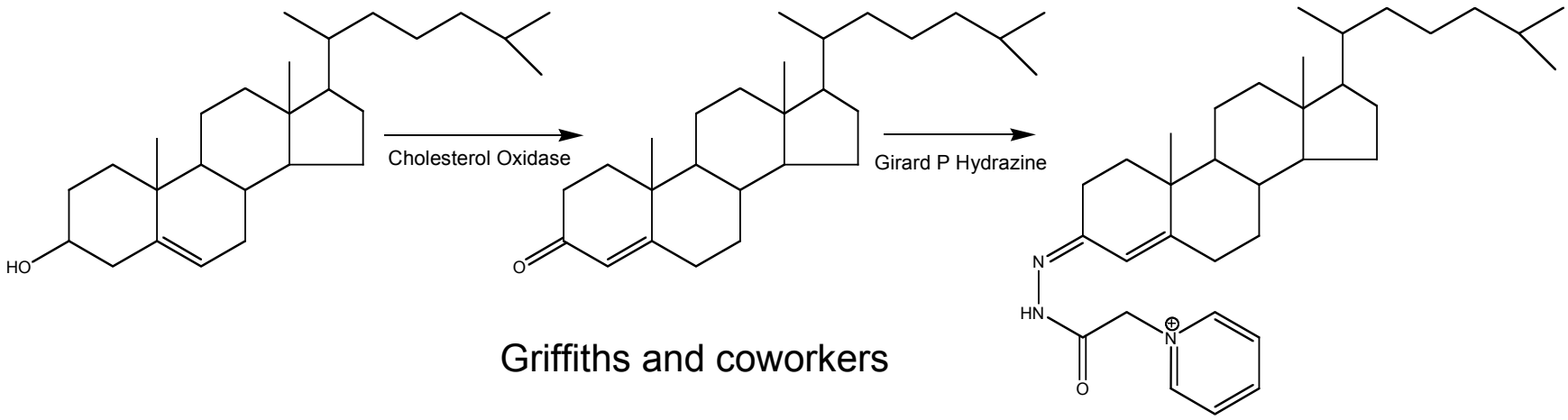
- Modern instruments
  - AB 5000
  - Scheduled MRMs
  - Biological relevance/sample size

Summary of Advanced Sterol Analysis

Process	Positive	Negative	Comment
base hydrolysis	Cleaner samples Less interference Higher quality data	Additional step Can degrade some sterols	Useful for free versus esterified analysis
SPE	Isolate oxysterols Remove interferences Cleaner samples	Expense and time Can't separate sterols	Split sample for GC- and LC-MS analysis
GC-MS	Best choice for sterols Inexpensive Ease of use	Detectability Limied injection vol. Derivitization	Can rival LC-MS with reduced cost and complexity
HPLC	Normal phase Alternative phases Speed	No single method provides complete resolution	Speed
MS	APCI better suited to sterols	Thermally labile compounds?	Dual mode sources availble

Identification of Novel Sterols

- No precursor, product, or neutral loss scans for native sterols
  - Oxidize 3β OH, derivatize with GP reagent



Griffiths and coworkers

- Use predicted MRM pairs
  - ~75 MRM pairs covers most logical sterols

Interesting Trends of Sterols in Macrophage Time Courses