Innovation Applied

Quantitation of Cholesterol Using Q-Exactive System

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Demonstration & Applications Laboratories
Life Sciences Mass Spectrometry

The work presented here is an outcome of a demonstration done for UCLA.

Sample info and demo criteria provided by UCLA

Sample Description:

- You will receive 21 unknown samples, one empty vial and 2 standard compounds/analytes to serve for tuning; compounds of interest to assay are Cholesterol and 26,26,26,27,27,27-d6 Cholesterol. The unknown samples, which are labeled 1-22, contain varying amounts of these products within a complex lipid matrix which is made of the neutral lipid extract (petroleum ether organic layer) of mouse brain.

Objective:

- Contained within the sample set is a standard curve. We would like you to provide us with the peak integration values for the two analytes, from which we will construct the curve and determine the accuracy with which the analytes spiked into matrix have been measured. Along with the data, we would ask for the chromatography method and mass spec settings you used for the analysis. Could you also please supply representative plots of the TIC, and representative plots of the selected ions used for quantitation? Lastly – we would also appreciate seeing the product ion spectra of the two analytes, as derived from the determined correct retention time, from a trap analysis.

Background:

- Our analysis of these samples was derived from McDonald *et. al.*,* with the following modifications: the dried lipid samples are dissolved in 200µl 90% methanol, 5mM ammonium acetate. Chromatography is achieved with a Phenomenex Synergi Fusion column 2X150mm, gradient between 85% and 99% methanol with 5mM ammonium acetate over a 4 minute period. 85% methanol with ammonium acetate is then passed over the column for (1) minutes to equilibrate the column for the next run. Mass Spec data were collected from the Applied Biosystems 4000 QTRAP mass spectrometer equipped with a TurboV electrospray ionization source in positive MRM mode. The total run time of the analysis is 6 minutes.

List of Cholesterol molecules

Sterol	LOD (fmoles)
22R-hydroxycholesterol	50
24-hydroxycholesterol	62
25-hydroxycholesterol	25
27-hydroxycholesterol	56
24,24-epoxycholesterol	19
7α-hydroxycholesterol	62
7-ketocholesterol	87
5β,6β-epoxycholesterol	5
5α,6α-epoxycholesterol	6
4β-hydroxycholesterol	25
desmosterol	2,000
7-dehydrocholesterol	520
cholestenone	390
lathosterol	1,300
cholesterol	1000
lanosterol	175
Cholestanol*	NA

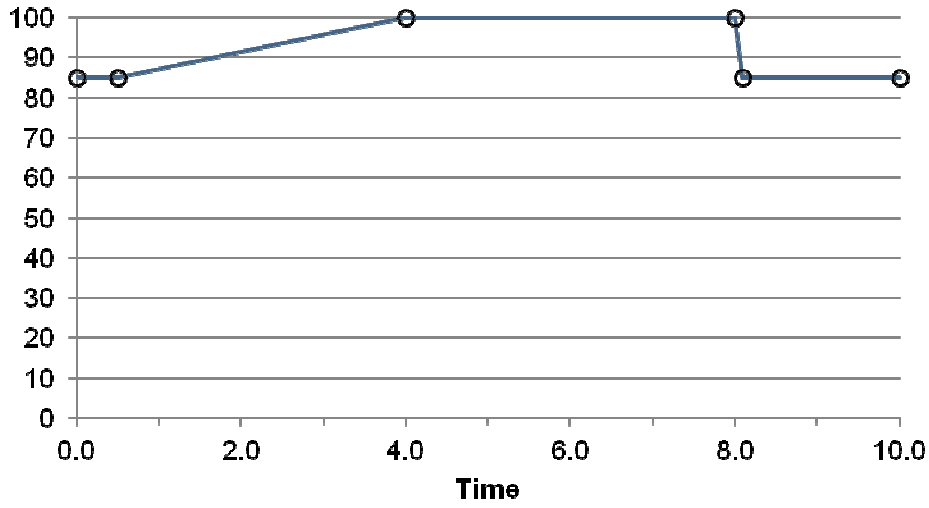
Table 2. Instrument Limits of Detection and Linear Range (mass on column)

Sterol	LOD (femtomoles)	Linear Range (femtomoles)
22R-hydroxycholesterol	50	$1.25e^{2}$ - $5.00e^{3}$
24-hydroxycholesterol	62	$1.50e^{2}$ - $6.50e^{3}$
25-hydroxycholesterol	25	$2.50e^{2}$ - $2.50e^{3}$
27-hydroxycholesterol	56	$1.25e^{2}$ - $5.50e^{3}$
24,24-epoxycholesterol	19	$1.25e^{2}$ - $2.00e^{3}$
7α-hydroxycholesterol	62	$1.25e^{2}$ - $6.00e^{3}$
7-ketocholesterol	87	$6.30e^{2}$ - $9.00e^{3}$
5β,6β-epoxycholesterol	5	$8.70e^{1}$ - 5.00
5α,6α-epoxycholesterol	6	$8.70e^{1}$ - 6.00
4β-hydroxycholesterol	25	$6.20e^{2}$ - $2.50e^{3}$
desmosterol	2,000	$3.20e^{4}$ - $6.50e^{4}$
7-dehydrocholesterol	520	$3.30e^{3}$ - $5.20e^{4}$
cholestenone	390	$1.60e^{3}$ - $3.50e^{4}$
lathosterol	1,300	$2.50e^{3}$ - $9.00e^{4}$
cholesterol	1000	$3.20e^{3}$ - $2.60e^{4}$
lanosterol	175	$3.00e^{3}$ - $2.30e^{4}$
Cholestanol*	NA	NA

Cholesterol – HPLC Analysis Conditions

- Thermo Hypersil Gold: 100x2.1mm, 1.9um
- Mobile Phase A: Water/5 mM Ammonium Acetate
- Mobile Phase B: Methanol/5mM Ammonium Acetate
- Flow Rate: 0.25 ml/min
- Injection:5 ul

Time	%A	%B
0.0	70	85
0.5	70	85
5.0	5	100
8	5	100
8.1	0	85
10	0	85

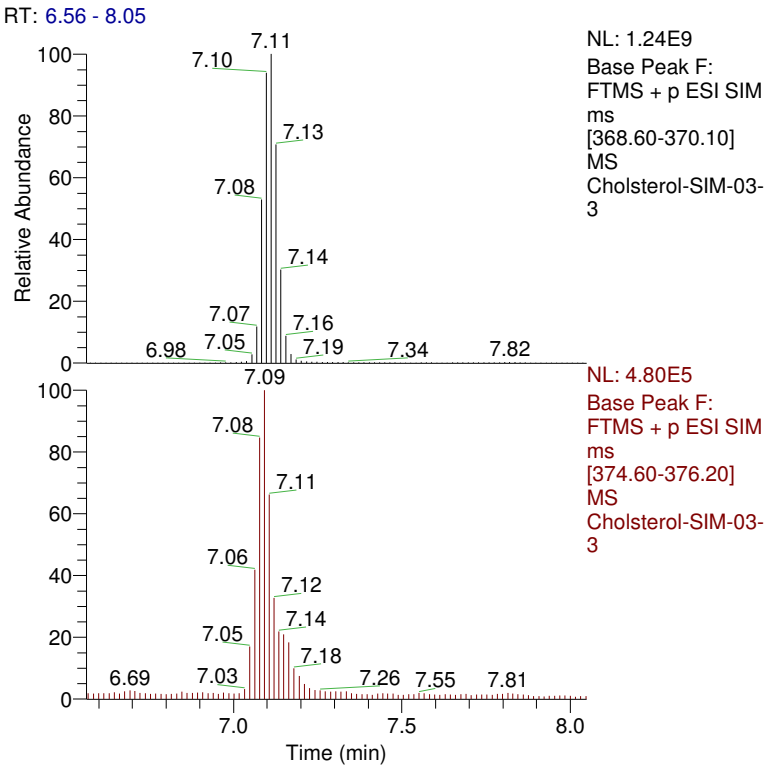


- 21 samples were submitted
- Samples were randomized and the sample key was unavailable until 2 weeks after data was reported

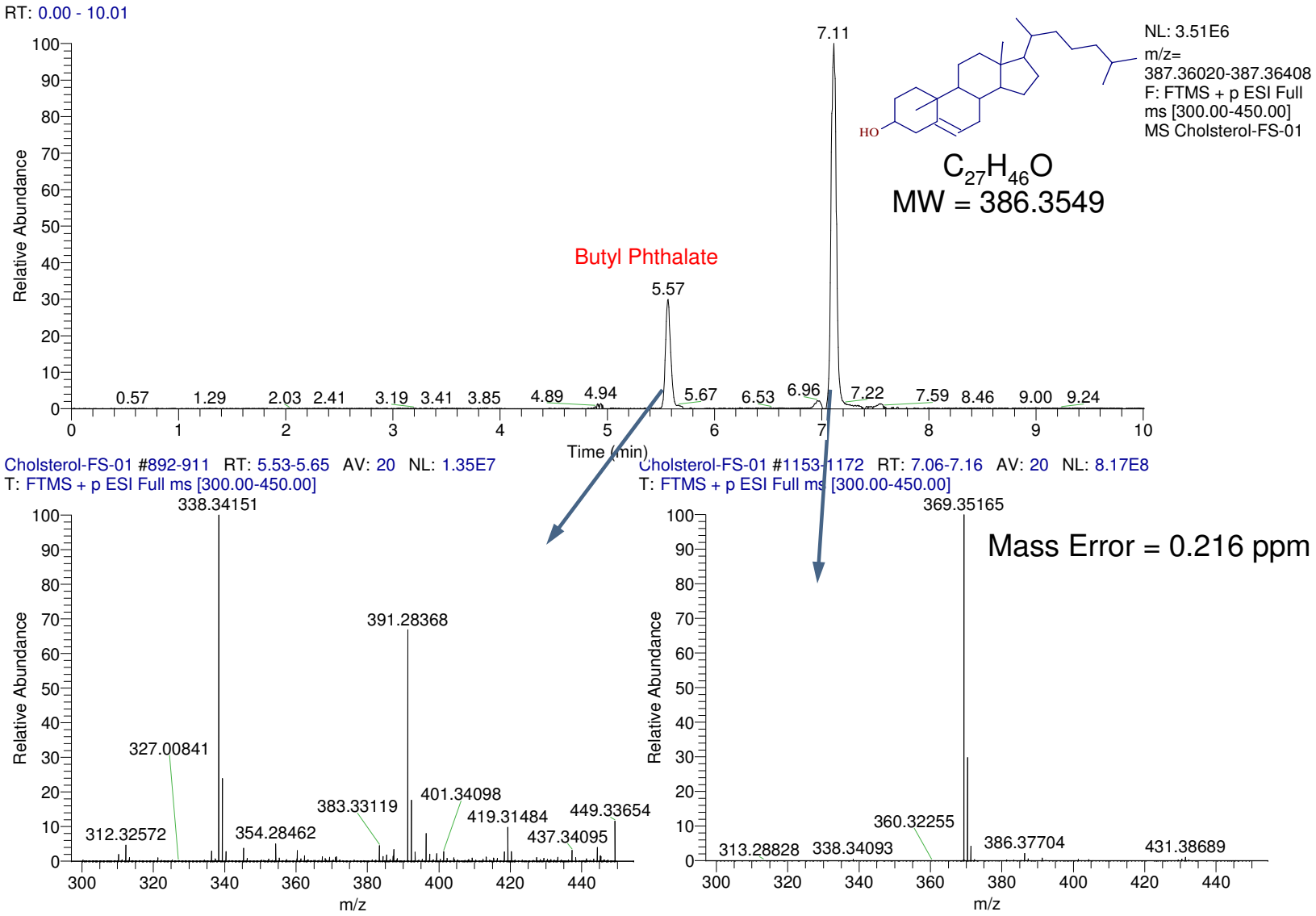
Q Exactive Settings

Method of Q Exactive

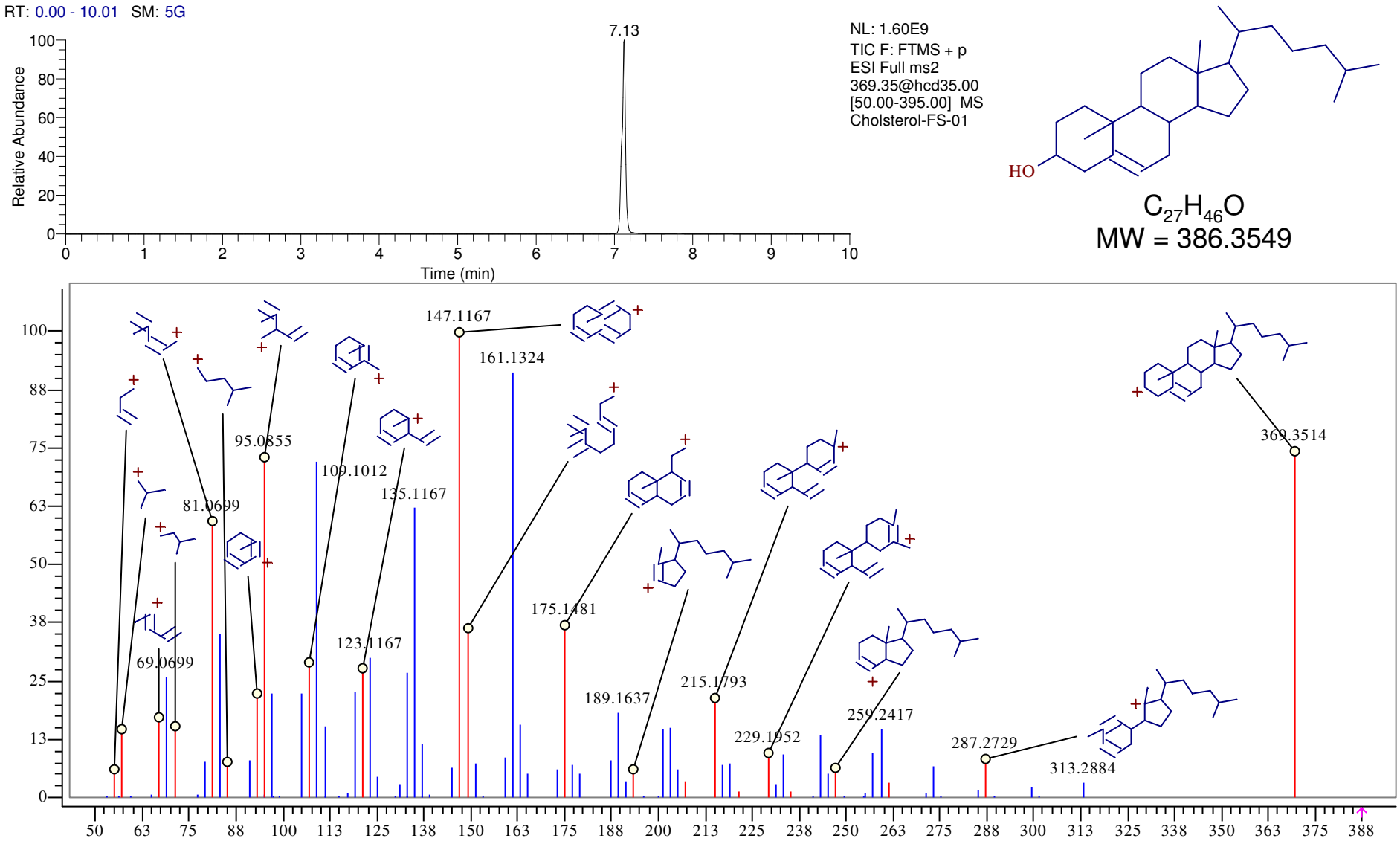
OVERALL METHOD SETTINGS	
Method duration	10.00 min
FULL MS — SIM	
General	
Runtime	0 to 10 min
Polarity	positive
In-source CID	0.0 eV
Full MS — SIM	
Microscans	1
Resolution	70,000
AGC target	3e6
Maximum IT	80 ms
Number of scan ranges	1
Scan range	368.6 to 370.1 m/z
FULL MS — SIM	
General	
Runtime	0 to 10 min
Polarity	positive
In-source CID	0.0 eV
Full MS — SIM	
Microscans	1
Resolution	70,000
AGC target	3e6
Maximum IT	250 ms
Number of scan ranges	1
Scan range	374.6 to 376.2 m/z



Cholesterol – Full Scan

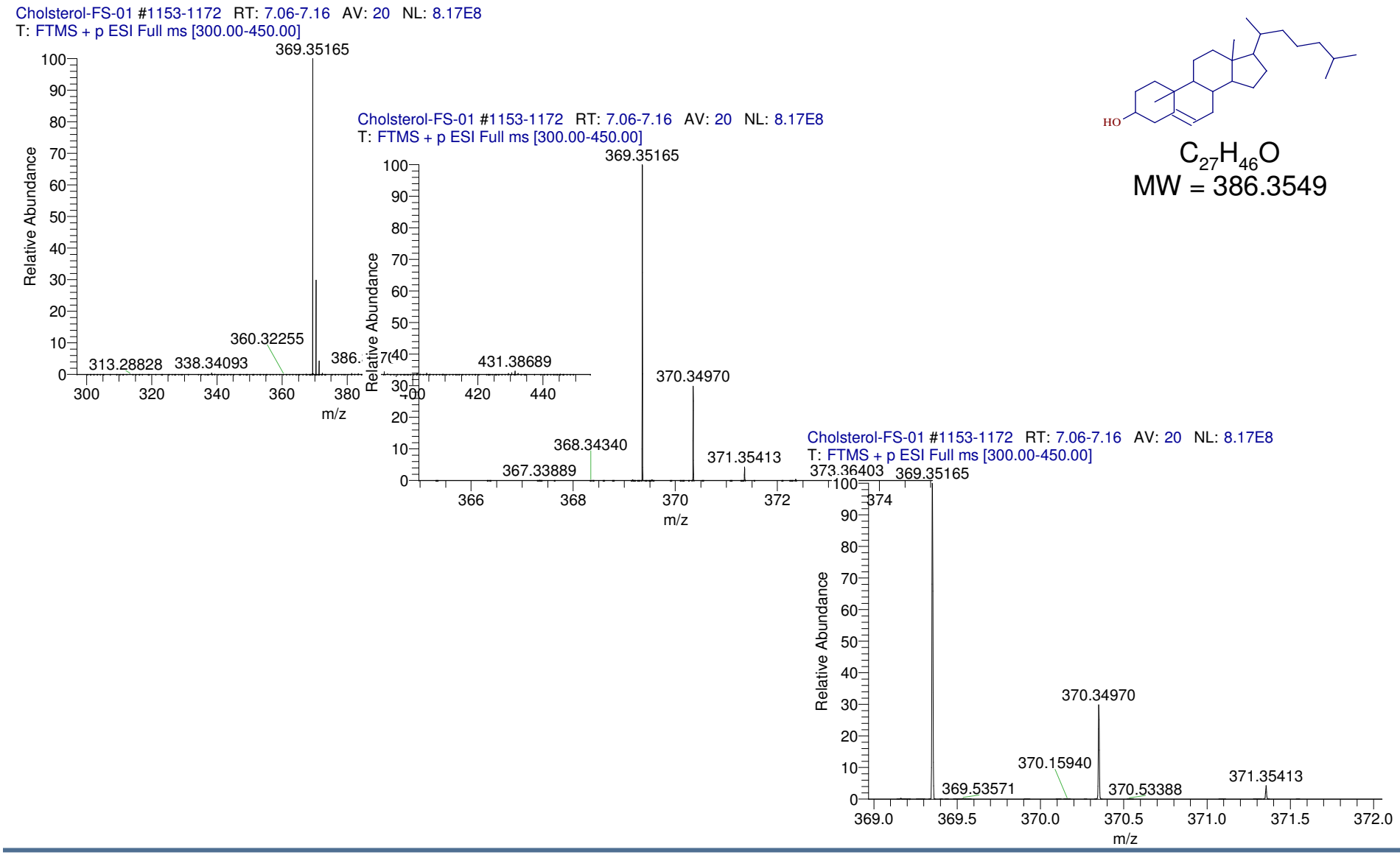


Cholesterol – HCD MS^2 Analysis Using Mass Frontier™



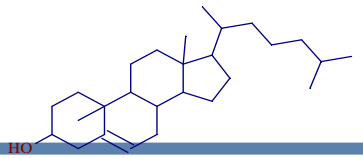
ThermoFisher SCIENTIFIC

Cholesterol – Full Scan R = 70,000 FWHM

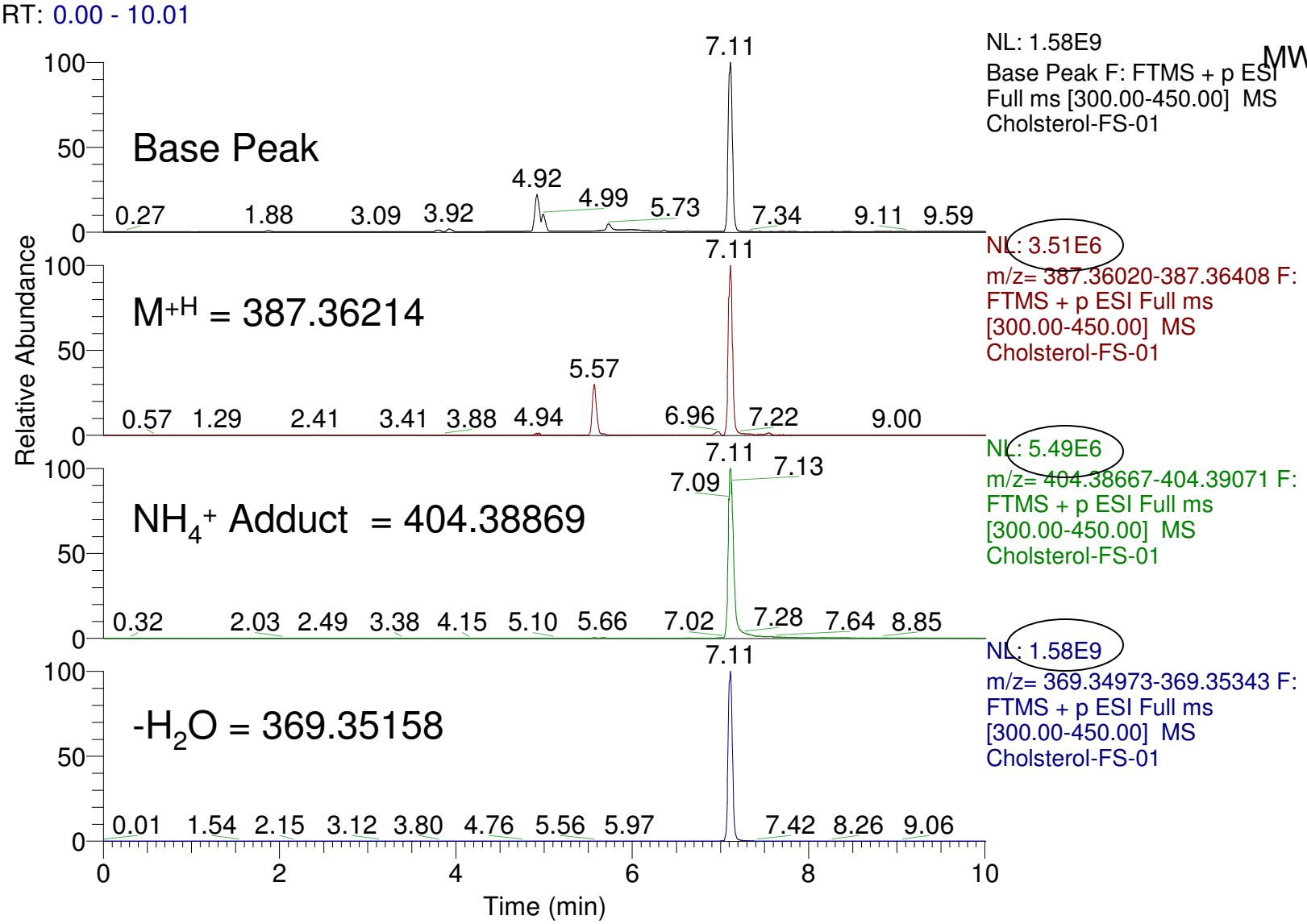


$C_{27}H_{46}O$
MW = 386.3549

Cholesterol – Full Scan, R = 70,000

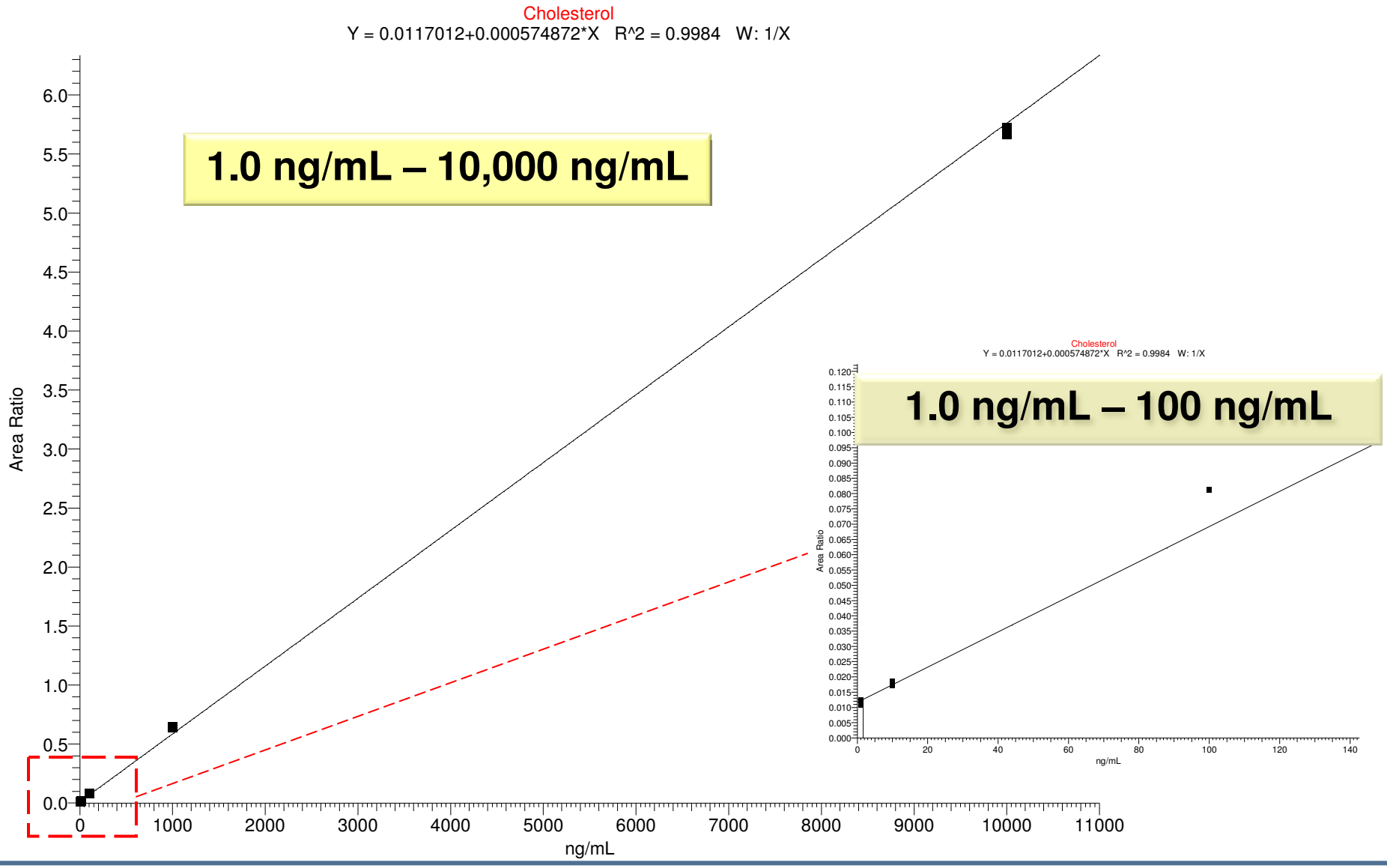


$C_{27}H_{46}O$
MW = 386.3549



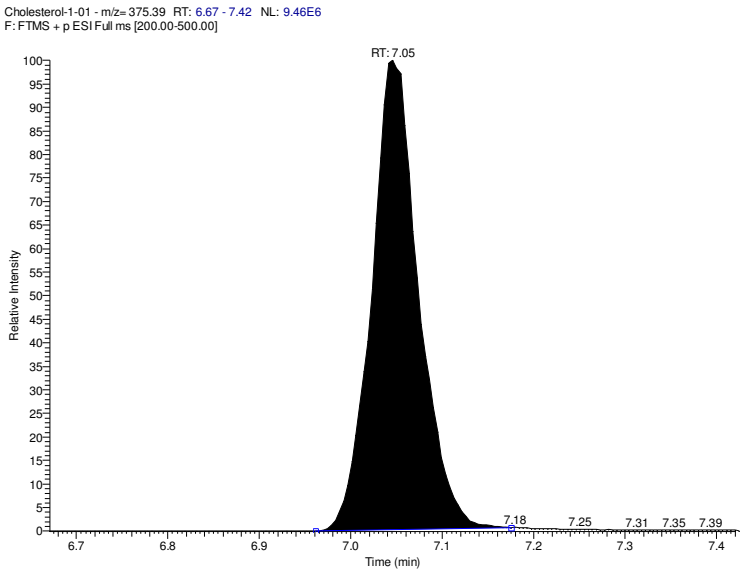
ThermoFisher SCIENTIFIC

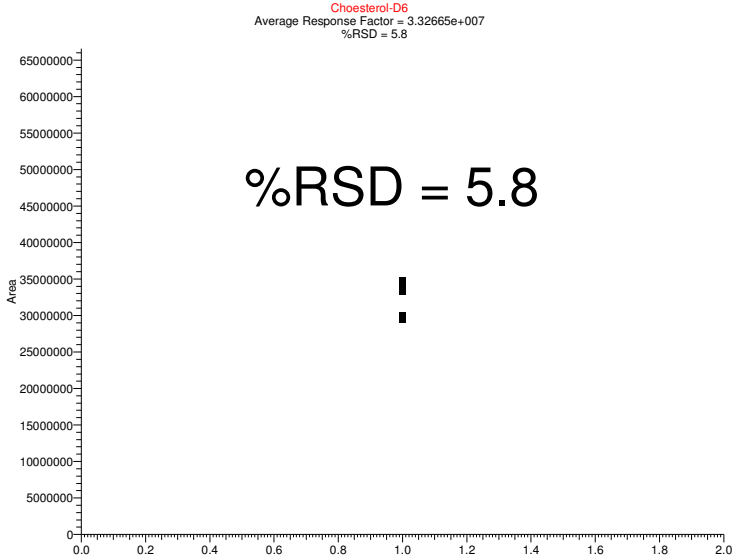
Cholesterol in Mobile Phase, Full-Scan (*m/z* 100- 500)



Internal Standard Reproducibility

Response	RT
33850969	7.05
34278125	7.05
34586537	7.04
34199157	7.04
34464670	7.05
34777514	7.04
34523675	7.04
34673829	7.05
34489027	7.05
33265654	7.05
33379819	7.05
33581548	7.05
34540263	7.05
34035111	7.05
33990347	7.05
34258630	7.05
29424660	7.03
29535160	7.04
29500160	7.04
29975379	7.04



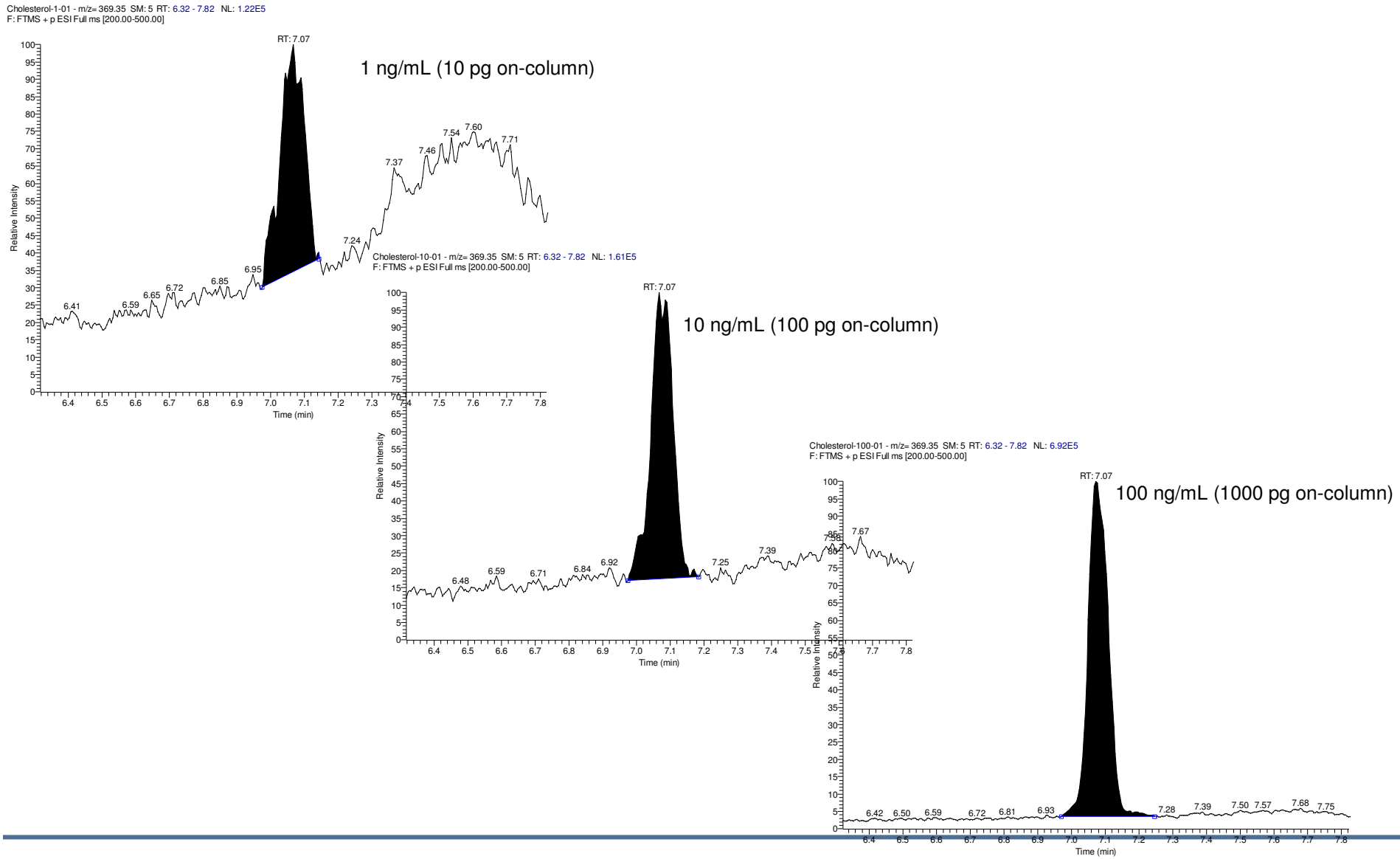


%RSD = 5.8

Cholesterol Standard Curve (10 µl Injection)

FileName	Sample Type	Response	ISTD Area	Response Ratio	Specified Conc (ng/mL)	Calculated Conc (ng/mL)	% CV	RT
Cholesterol-1-01	Standard	403827	33850969	0.012	1	0.77	0.730	7.07
Cholesterol-1-02	Standard	407759	34278125	0.012	1	0.71	0.730	7.06
Cholesterol-1-03	Standard	407023	34586537	0.012	1	0.49	0.730	7.06
Cholesterol-1-04	Standard	402234	34199157	0.012	1	0.48	0.730	7.05
Cholesterol-10-01	Standard	612299	34464670	0.018	10	10.92	0.100	7.07
Cholesterol-10-02	Standard	616968	34777514	0.018	10	10.88	0.100	7.06
Cholesterol-10-03	Standard	613369	34523675	0.018	10	10.92	0.100	7.05
Cholesterol-10-04	Standard	614889	34673829	0.018	10	10.86	0.100	7.07
Cholesterol-100-01	Standard	2809482	34489027	0.081	100	121.71	0.200	7.07
Cholesterol-100-02	Standard	2705676	33265654	0.081	100	121.49	0.200	7.06
Cholesterol-100-03	Standard	2711165	33379819	0.081	100	121.29	0.200	7.07
Cholesterol-100-04	Standard	2722750	33581548	0.081	100	121.04	0.200	7.07
Cholesterol-1000-01	Standard	22306713	34540263	0.646	1000	1103.30	0.440	7.07
Cholesterol-1000-02	Standard	21893027	34035111	0.643	1000	1098.83	0.440	7.07
Cholesterol-1000-03	Standard	21830428	33990347	0.642	1000	1097.10	0.440	7.07
Cholesterol-1000-04	Standard	21892673	34258630	0.639	1000	1091.52	0.440	7.07
Cholesterol-10000-01	Standard	168374771	29424660	5.722	10000	9932.80	0.430	7.07
Cholesterol-10000-02	Standard	168328534	29535160	5.699	10000	9892.84	0.430	7.07
Cholesterol-10000-03	Standard	167602499	29500160	5.681	10000	9861.80	0.430	7.07
Cholesterol-10000-04	Standard	169827920	29975379	5.666	10000	9834.26	0.430	7.07
Blank-04	Blank	5042	NF	NC	NA	NC	NA	7.05

Cholesterol Chromatograms

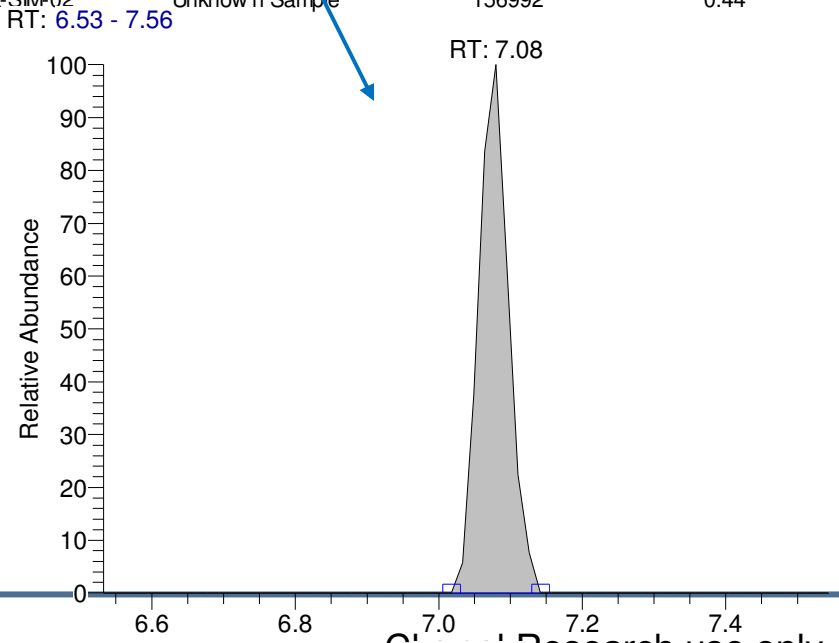


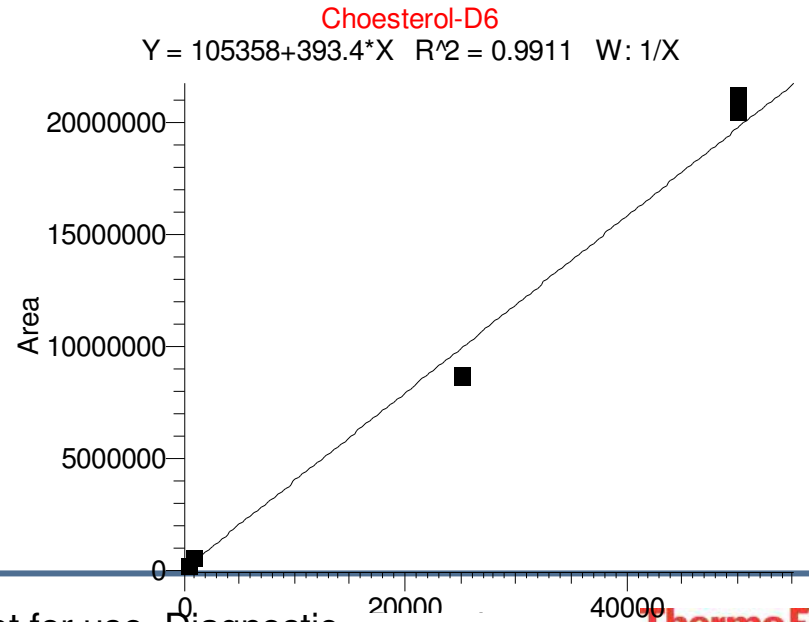
Cholesterol-D6 – UCLA Dilutions, No Matrix

Y = 1.36895e+006+670.119*X R^2 = 0.9956

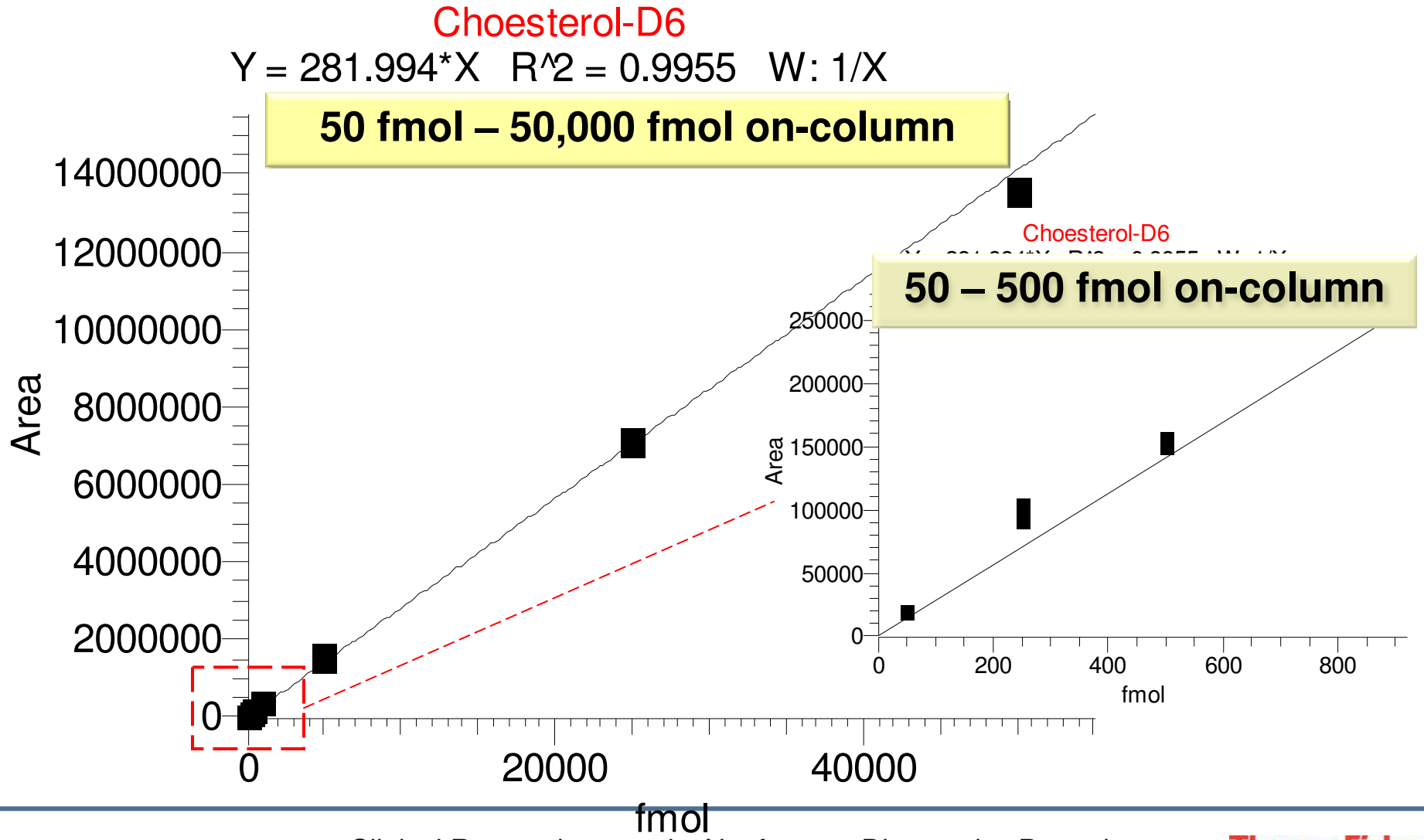
Component Name	Origin Index
Cholesterol	Ignore

Filename	Sample Type	Area	Specified Amount	Calculated Amount	%RSD-AMT	Level	RT
Cholsterol-SIM-18-1	Std Bracket Sample	1722671	500.000	527.849	19.7%	6	7.10
Cholsterol-SIM-18-2	Std Bracket Sample	1605773	500.000	353.405	19.7%	6	7.10
Cholsterol-SIM-18-3	Std Bracket Sample	1667274	500.000	445.182	19.7%	6	7.10
Cholsterol-SIM-19-1	Std Bracket Sample	2136121	1000.000	1144.829	5.3%	5	7.10
Cholsterol-SIM-19-2	Std Bracket Sample	2109763	1000.000	1105.496	5.3%	5	7.10
Cholsterol-SIM-19-3	Std Bracket Sample	2190912	1000.000	1226.593	5.3%	5	7.10
Cholsterol-SIM-20-1	Std Bracket Sample	16454977	25000.000	22512.467	2.1%	2	7.10
Cholsterol-SIM-20-2	Std Bracket Sample	17101086	25000.000	23476.639	2.1%	2	7.10
Cholsterol-SIM-20-3	Std Bracket Sample	16664256	25000.000	22824.769	2.1%	2	7.10
Cholsterol-SIM-21-1	Std Bracket Sample	37115795	50000.000	53344.043	2.3%	1	7.10
Cholsterol-SIM-21-2	Std Bracket Sample	35837018	50000.000	51435.759	2.3%	1	7.10
Cholsterol-SIM-21-3	Std Bracket Sample	35614010	50000.000	51102.970	2.3%	1	7.10
Blank-SIM-01	Unknow n Sample	379694	1.07	-1476.238	NA	NA	7.12
Blank-SIM-02	Unknow n Sample	156992	0.44	-1808.571	NA	NA	7.10

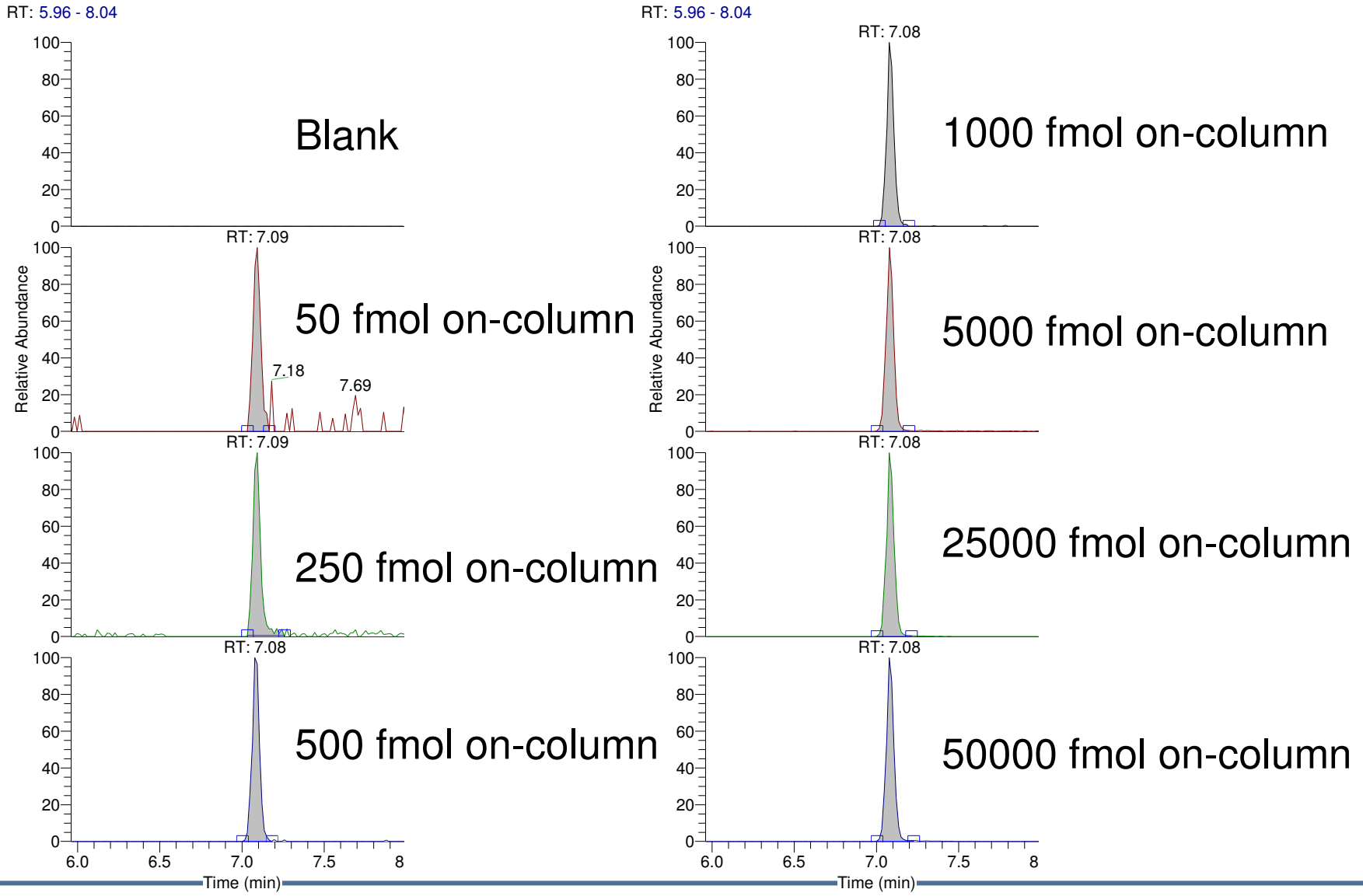




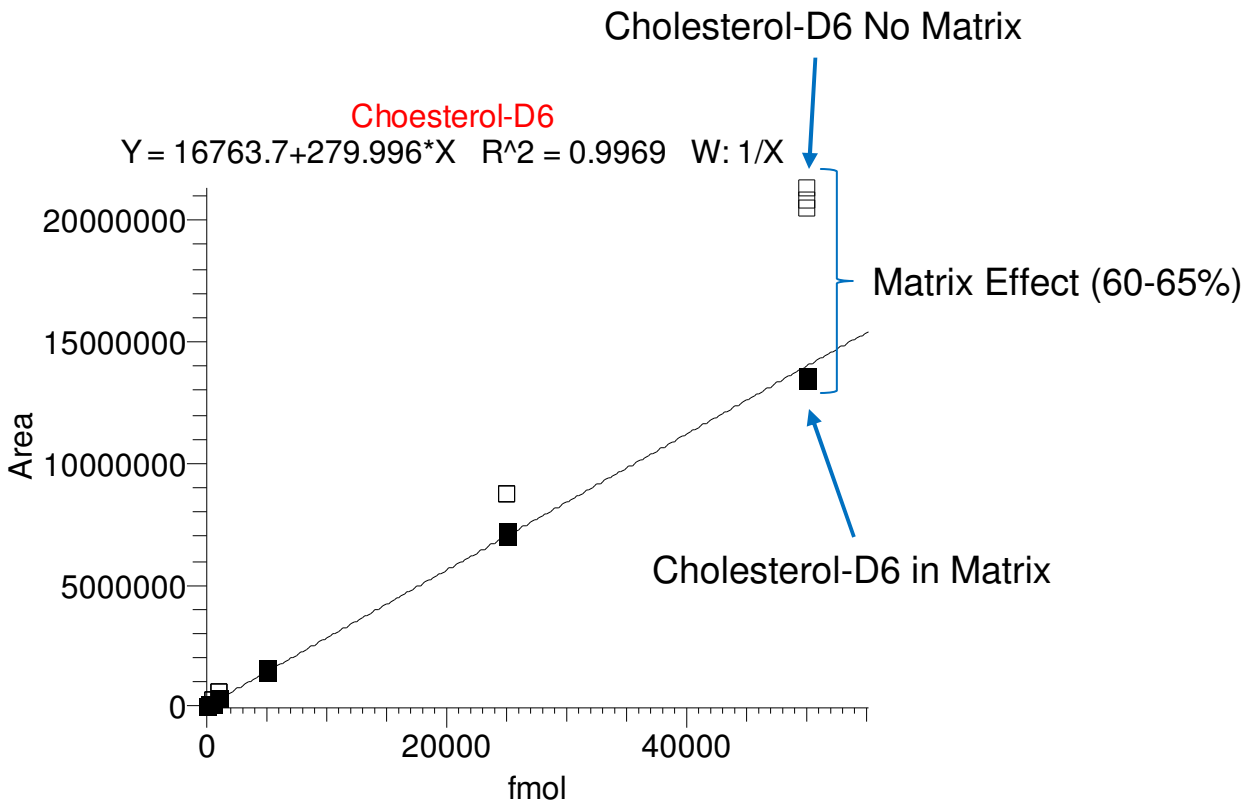
Cholesterol-D6 UCLA Dilutions in Matrix, SIM Scan



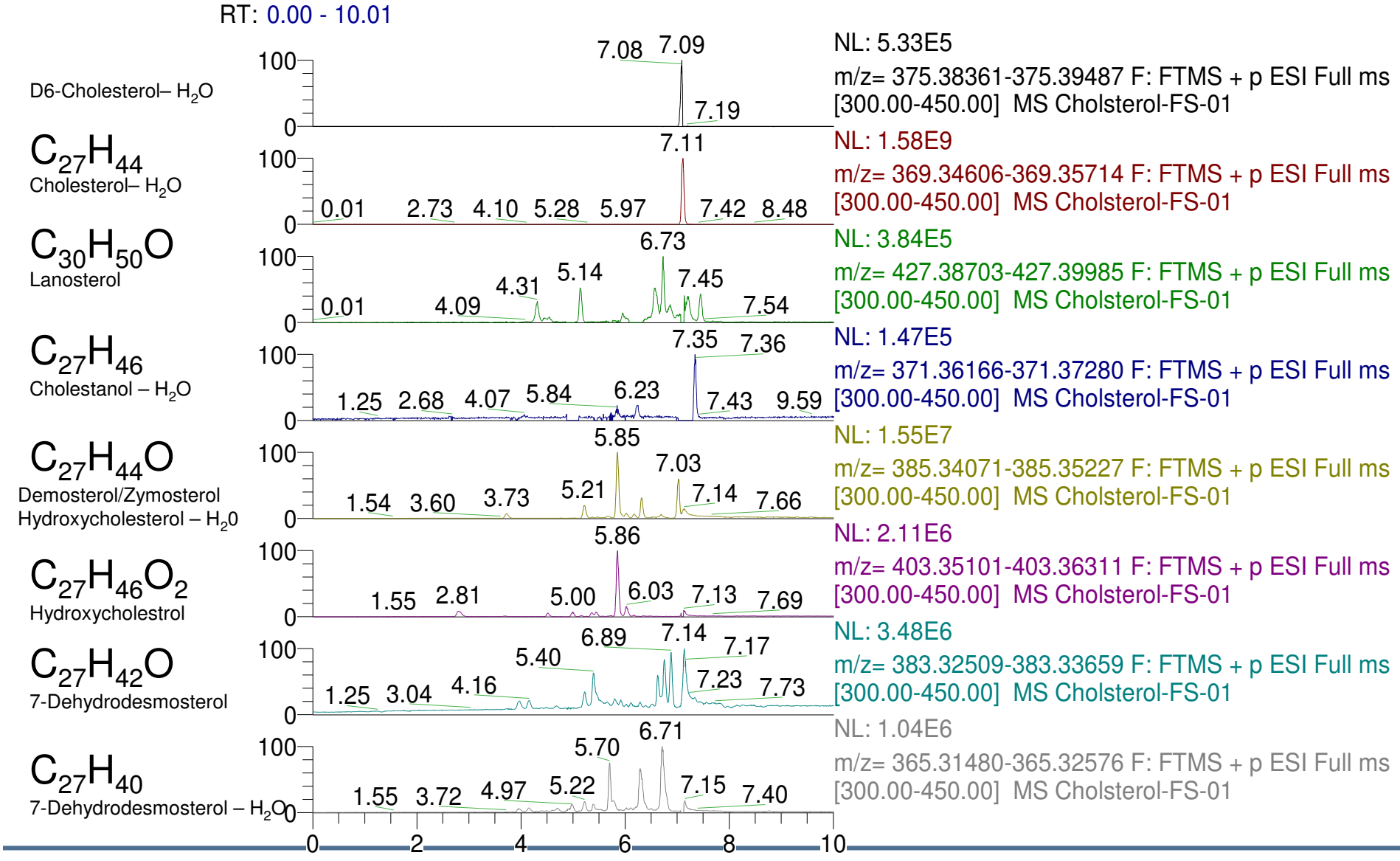
Cholesterol-D6 UCLA Dilutions in Matrix, Chromatograms



Matrix Effect – UCLA dilutions



XIC of Various Cholesterol forms - 5 ppm window



Summary

- Using High Resolution/Accurate Mass SIM analysis, Cholesterol-D6 is linear from 50 to 50000 fmol on-column in the presence of high levels of Cholesterol. It is possible that the LOD is lower, but standards at 1 and 5 fmol/ul were not included.

- Alternate forms of Cholesterol can also be identified in the sample using Full Scan analysis (slide 27).

- The Q Exactive can clearly identify the difference between ^{13}C and ^{15}N labeled molecules (slide 11).

- There is an effect of high levels of Cholesterol on the analysis of high levels of Cholesertol-D6 (slide 27), but the curve remains linear over four orders of magnitude and the LOQ is 50 fmol on-column, possibly lower.

- Appendix

Development of a Rapid and Sensitive Method for Sterol Analysis by HPLC-ESI-MS

Erin C. McCrum; Jeffery G. McDonald

The University of Texas Southwestern Medical Center, Dallas, Texas

Overview

PURPOSE: Develop an HPLC-ESI-MS method for the analysis of sterols

METHODS: Utilize RP-HPLC (MeOH/H_2O) to resolve sterols and ESI-MS for detection

RESULTS: Common sterols are well resolved with a water/methanol gradient; incorporating ammonium acetate results in sterol adduct formation, and excellent sensitivity is achieved

Introduction

Sterols pose unique challenges with respect to analytical measurement, both directly and in conjunction with chromatographic systems. Sterols lack chromophores and consequently are not well detected by UV-Vis spectroscopy. Sterol analysis by GC-MS requires derivitization of the alcohol group prior to analysis. Furthermore, GC-MS typically does not allow for advanced mass spectral study of sterols such as MS/MS experiments. The development of ESI has resulted in HPLC-MS becoming a widely available analytical tool in many research laboratories. However, sterol analysis by HPLC-ESI-MS has been historically under-utilized because of the difficulty in ionizing low polarity sterols. We present here a rapid and sensitive HPLC-ESI-MS method for sterol analysis by utilizing ammonium adducts.

Methods

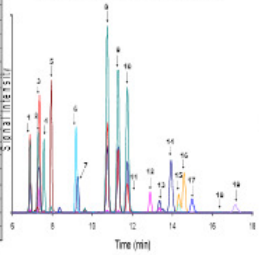
- Sterols were resolved by reverse-phase HPLC using a binary solvent system consisting of: solvent A (methanol) and solvent B (water); both with 5mM ammonium acetate.
- Gradient elution was performed on a Luna C_{18} HPLC column (2 × 150 mm, 3 µm particle size; Phenomenex, Torrance, CA) at a flow rate of 0.25 mL/min with 10 µl injections
- Solvent A was held at 85% for 1 min; increased to 100% A in 5 min; was maintained at 100% A for 10.5 min; and then re-equilibrated to the starting conditions for 5 min.
- The HPLC was coupled to a 4000 QTrap triple quadrupole MS (Applied Biosystems, Foster City, CA) operated in positive mode and equipped with a TurboV ESI source.
- The MS curtain gas was 15 psi, gas 1 was 80 psi (50 °C) and gas 2 was 20 psi; spray voltage was 5500 V, and collision gas was set to medium.
- Selected reaction monitoring transitions (SRM) were optimized with respect to declustering potential and collision energy for each sterol of interest.

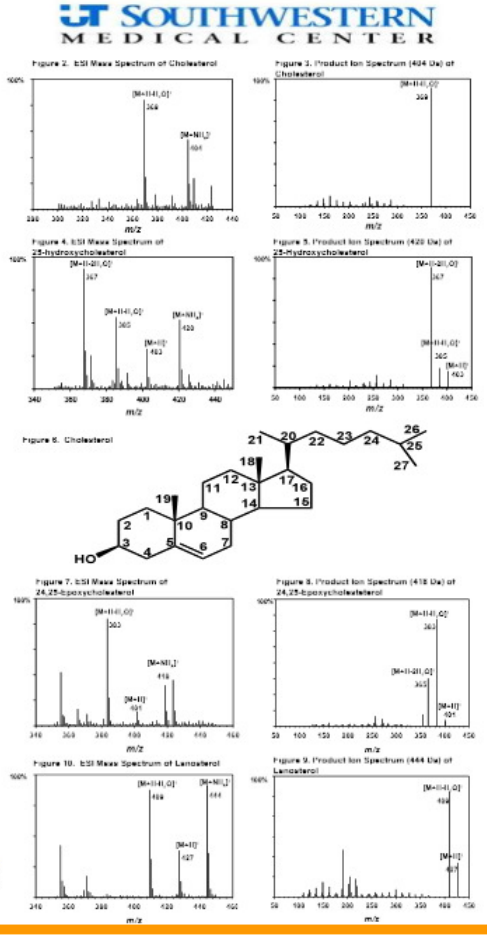
Table 1. Selected Reaction Monitoring (SRM) pairs of common sterols

Sterol	Peak # (Fig. 1)	SRM Ion Pair (Da)
22R-hydroxycholesterol	1	420/355
24-hydroxycholesterol	2	420/355
25-hydroxycholesterol	3	420/367
27-hydroxycholesterol	4	420/355
24,24-epoxycholesterol	5	418/383
7α-hydroxycholesterol	6	385/367
7-ketocholesterol	7	401/382
5β,6β-epoxycholesterol	8	420/355
5α,6α-epoxycholesterol	9	420/355
4β-hydroxycholesterol	10	420/355
zymosterol*	11	385/367
desmosterol	12	402/367
7-dehydrocholesterol	13	385/367
cholestenone	14	385/367
lathosterol	15	404/369
cholesterol	16	404/369
lanosterol	17	444/409
cholestanol	18	404/387
24-dihydrolanosterol	19	429/411

* zymosterol is no longer commercially available, but is known to elute between 4β-hydroxycholesterol and desmosterol

Figure 1. HPLC-ESI-MS Chromatogram (SRM) of a mixture of common sterols





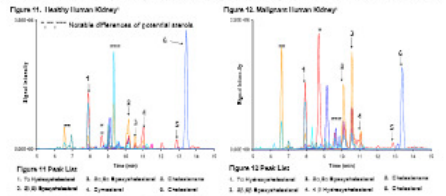
Results

Table 2. Instrument Limits of Detection and Linear Range (mass on column)

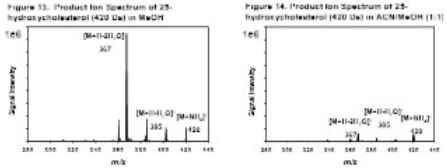
Sterol	LOD (femtomoles)	Linear Range (femtomoles)
22R-hydroxycholesterol	50	$1.25e^2$ - $5.00e^3$
24-hydroxycholesterol	62	$1.50e^2$ - $6.50e^3$
25-hydroxycholesterol	25	$2.50e^2$ - $2.50e^3$
27-hydroxycholesterol	56	$1.25e^2$ - $5.50e^3$
24,24-epoxycholesterol	19	$1.25e^2$ - $2.00e^3$
7α-hydroxycholesterol	62	$1.25e^2$ - $6.00e^3$
7-ketocholesterol	87	$6.30e^2$ - $9.00e^3$
5β,6β-epoxycholesterol	5	$8.70e^2$ - 5.00
5α,6α-epoxycholesterol	6	$8.70e^2$ - 6.00
4β-hydroxycholesterol	25	$6.20e^2$ - $2.50e^3$
desmosterol	2,000	$3.20e^3$ - $6.50e^3$
7-dehydrocholesterol	920	$3.30e^3$ - $5.20e^3$
cholestenone	390	$1.60e^3$ - $3.90e^3$
lathosterol	1,300	$2.50e^3$ - $9.00e^3$
cholesterol	1000	$3.20e^3$ - $2.60e^4$
lanosterol	175	$3.00e^3$ - $2.30e^4$
Cholestanol*	NA	NA

* Standard was determined to be impure and limit of detection not accurately determined

HPLC-ESI-MS (SRM) Analysis of Healthy and Malignant Human Kidney Extracts



Effect of Acetonitrile on Signal Intensity



Conclusions

- HPLC-ESI-MS is well suited for the analysis of sterols when ammonium adducts are utilized
  - A water/methanol solvent system resolves most common sterols on a C_{18} HPLC column
  - Ammonium adducts of sterols exhibit excellent sensitivity in SRM mode
- Incorporating acetonitrile causes a significant decrease in signal intensity
- Isomeric nature of sterols yields common mass peaks and SRM pairs which can both aid and hinder sterol analysis

Acknowledgments

Matthew Merrit' ; NIH Glue Grant 1 U54 GM69338; David Russell, PI Lipid MAPS Sterol Core; lipidmaps.org

References

Puffer, M.K., Murphy, R.C. (2004) J. Biol. Chem. 279, 28331.