Applying UHPLC-HRAM MS technology to characterize and quantify lipid components in support of LNP development and quality control

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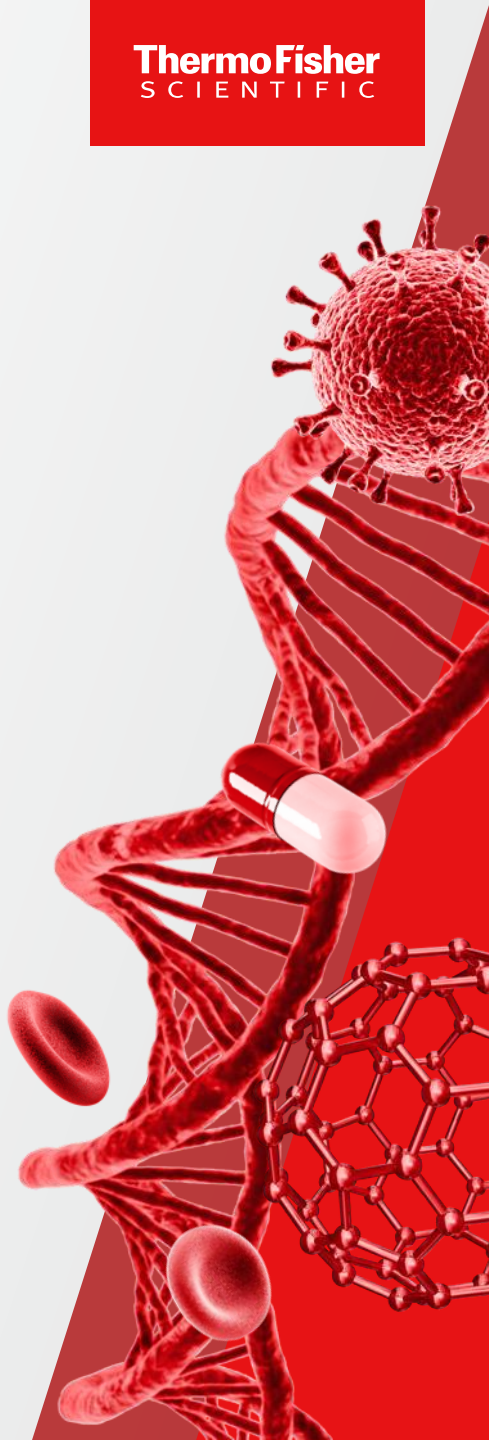
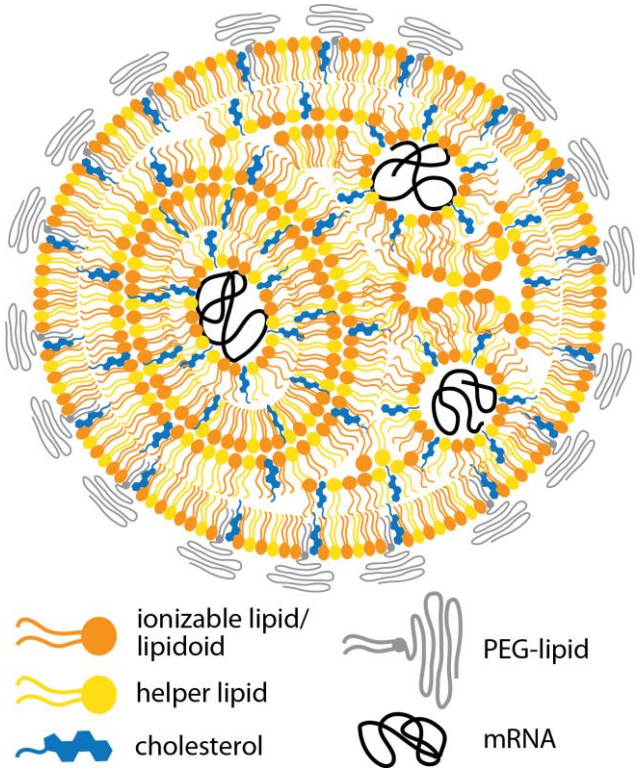
Pharma/Biopharma Vertical Marketing
April 2023



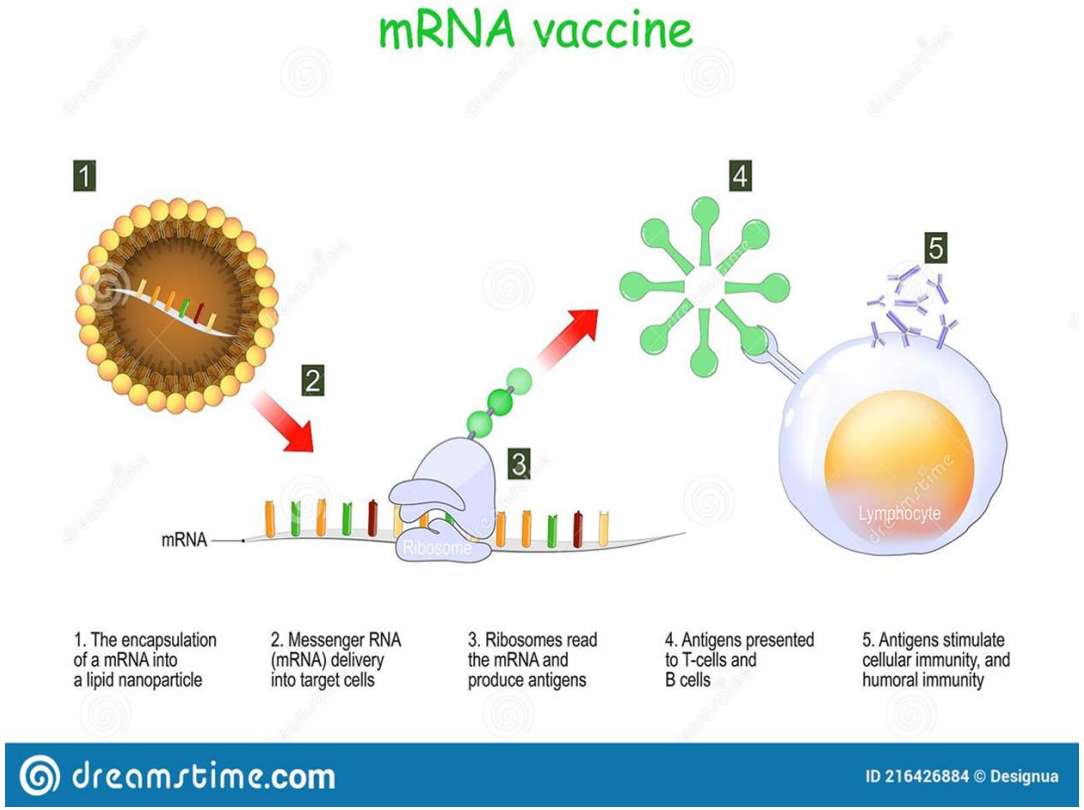
The world leader in serving science

lipid nanoparticles (LNPs): promising vehicles to deliver nucleic acids like DNA, mRNA, and siRNA



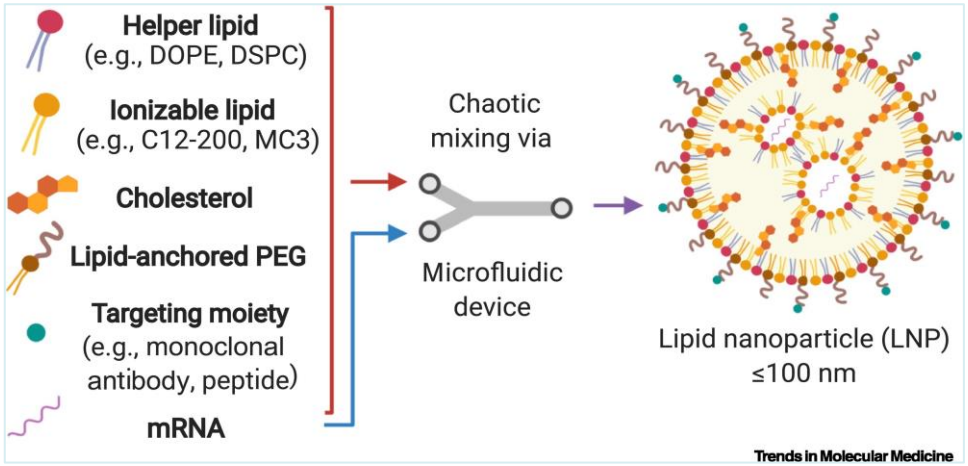
https://mitnano.mit.edu/events/mitnano-seminar-kathryn-whitehead

Used as mRNA carriers in both Pfizer-BioNTech and Moderna mRNA vaccines for COVID-19



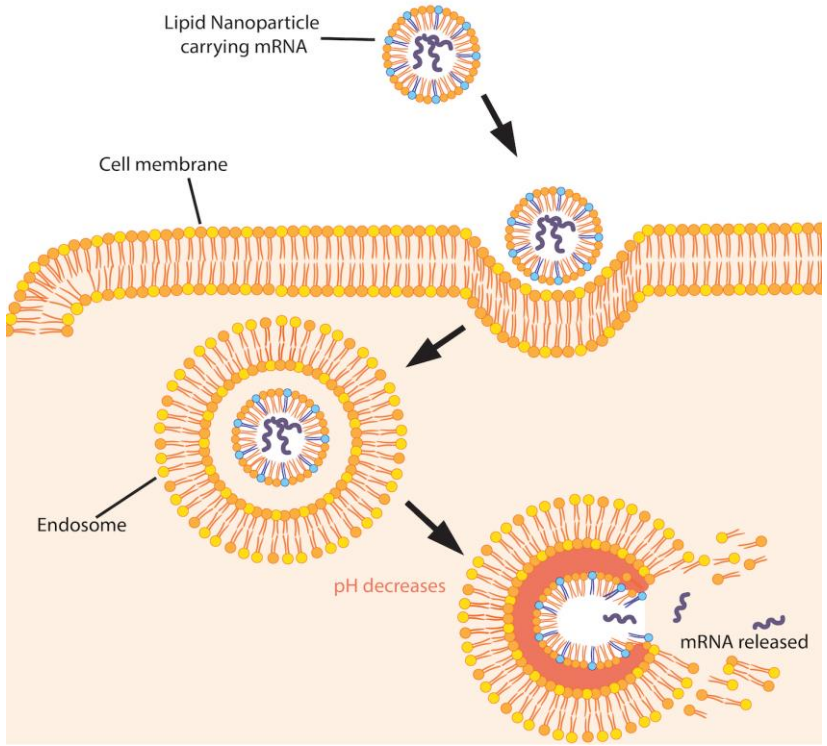
What are lipid nanoparticles (LNPs)

- LNPs are lipid-based drug delivery systems that carry nucleic acid material. These systems primarily rely on four lipid components: a PEG lipid, amino (cationic) lipid, structural lipid, and cholesterol.



https://doi.org/10.1016/j.molmed.2021.03.003

- LNP lipid components play important rules to encapsulate mRNA, protect it from destructive enzymes, and transport it into cells, where the mRNA is released and used to make proteins.



https://blog.scienceborealis.ca/lipid-nanoparticles-the-underrated-invention-behind-the-vaccine-revolution/

Importance to test the lipid identity and purity of LNPs

ThermoFisher SCIENTIFIC

Liposome Drug Products

Chemistry, Manufacturing, and Controls; Human Pharmacokinetics and Bioavailability; and Labeling Documentation

Guidance for Industry

Additional copies are available from:
Office of Communications, Division of Drug Information
Center for Drug Evaluation and Research
Food and Drug Administration
10001 New Hampshire Ave., Hillandale Bldg., 4th Floor
Silver Spring, MD 20993-0002
Phone: 855-543-3784 or 301-796-3400; Fax: 301-431-6353
Email: druginfo@fda.hhs.gov
http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/default.htm

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Drug Evaluation and Research (CDER)

April 2018
Pharmaceutical Quality/CMC

c. Specifications for Lipid Components

You should provide the following specification information for each lipid component used to manufacture the drug product.

i. The identity test capable of distinguishing the intended lipid component from lipids with similar structures.
ii. The assay based on a stability-indicating analytical procedure.
iii. The validated analytical procedures accompanied by the validation data.
iv. Impurity testing:
    1. Trans-fatty acid
    2. Free-fatty acid
    3. Peroxides (associated with unsaturated fatty acids)
    4. Lysophospholipids
    5. Solvents and catalysts used in the synthesis or purification processes
v. Other testing:
    1. Counterion content and limits on divalent cations, when appropriate
    2. The degree of unsaturation of the fatty acid side chains (for lipid mixtures)

Information about impurities, including synthetic by-products, where applicable, should be provided. Impurities may warrant identification and qualification, depending on the following:

i. The amount of the impurity in the final liposome drug product
ii. Known toxicities of the impurity
iii. Structural alerts[12]

Importance to test the lipid identity and purity of LNPs

ThermoFisher SCIENTIFIC

Liposome Drug Products

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iv. Impurity testing:
    1. Trans-fatty acid
    2. Free-fatty acid

Require analytical tools which can

- ❑ **Identify the lipid structure at molecular level with high confidence.**
- ❑ **Detect and characterize lipid impurities with high sensitivity and selectivity.**

ing:

i. The amount of the impurity in the final liposome drug product
ii. Known toxicities of the impurity
iii. Structural alerts[12]

Importance to quantify the LNP lipids and profile their metabolites in vivo from biological matrix samples

- Among the key LNP lipid components, the ionizable lipid plays a central role in nucleic acid material delivery efficacy. Since ionizable lipids are synthetic components, they should be rapidly degraded into non-toxic metabolites after successful intracellular cargo delivery to avoid immune responses and toxicity mediated by lipids.
- A key research area for new LNPs development is to develop the novel, next-generation ionizable lipids that combine the excellent nucleic acid delivery efficacy with biodegradable functionality leading to rapid elimination in vivo.
- For new LNPs development, researchers need to rapidly monitor the bio-degradability of the novel ionizable lipids after the LNP administration and identify their metabolites through the bio transformation from the biological matrix samples.

Importance to quantify the LNP lipids and profile their metabolites in vivo from biological matrix samples

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- For the nov he bio

Require analytical tools which can

- ❑ **Quantify the ionizable lipids and other synthetic lipids with high sensitivity and selectivity and simultaneously identify the associated lipid metabolites with high confidence from various tissue, plasma samples to support new-generation LNPs development and pre-clinical studies.**

Addressing the LNP analytical challenges using LC MS-MS/MS approach

Lipid raw material

LNP product

Biological matrix

(tissue, blood…)

Lipid extraction

HPLC –MS - MS/MS analysis

Thermo Scientific™ Vanquish™ UHPLC

Thermo Scientific™ Orbitrap Exploris™ mass spectrometers

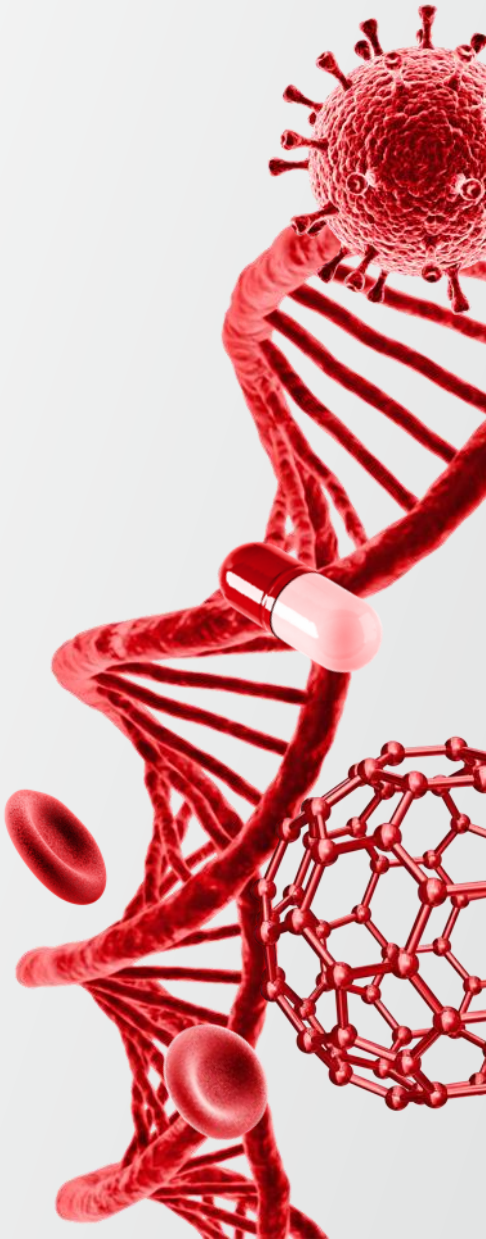
Data processing & reporting

Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software

Thermo Scientific™ Compound Discoverer™ software

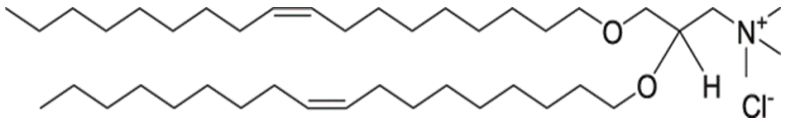
Thermo Scientific™ LipidSearch™ software

LC-MS-MS/MS method development using commercially available lipid references

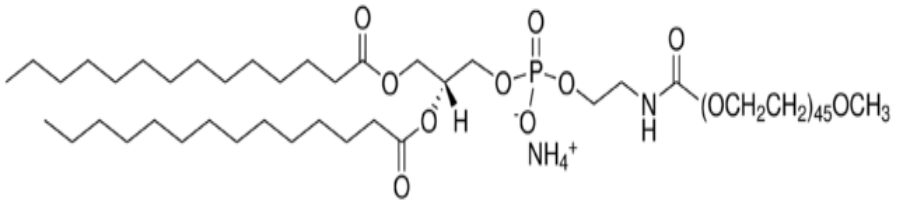


Lipid components used for LC MS-MS/MS method development

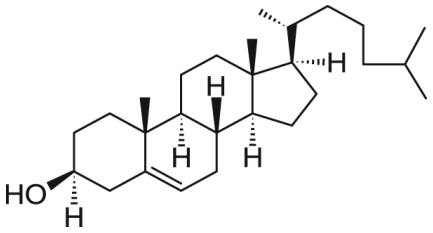
DOTMA: 1,2-di-O-octadecenyl-3-trimethylammonium propane (chloride salt)



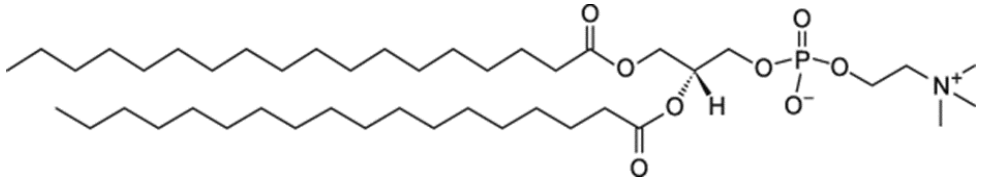
14:0 PEG 2000: 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt)



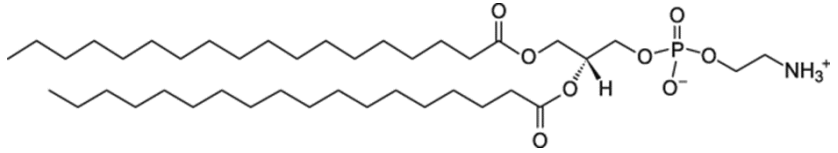
Cholesterol



18:0 PC (DSPC): 1,2-distearoyl-sn-glycero-3-phosphocholine



18:0 PE (DSPE): 1,2-distearoyl-sn-glycero-3-phosphoethanolamine



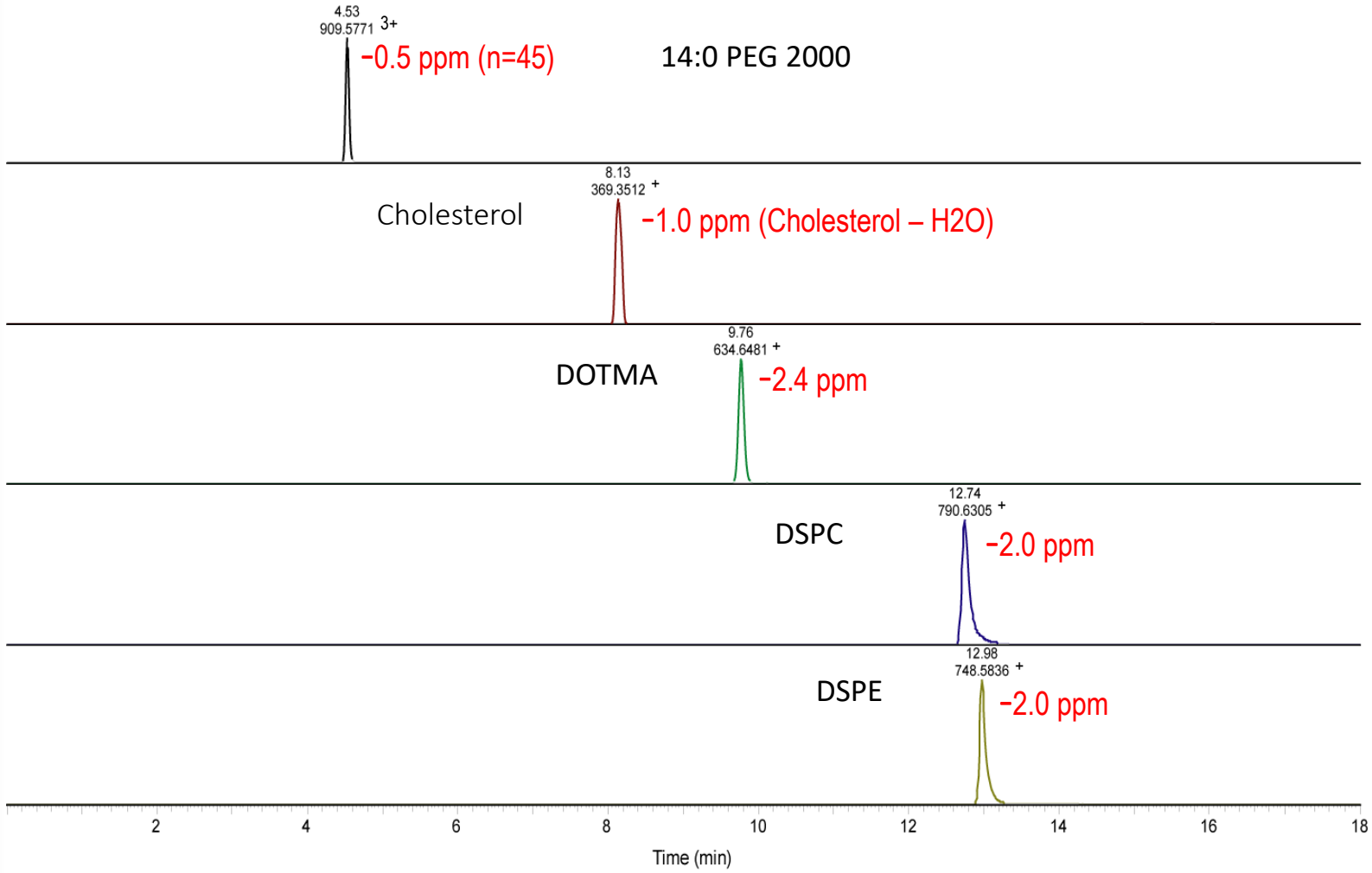
Rapid lipid identification using LC MS-MS/MS

Vanquish H system

- Thermo Scientific™ Accucore™ C30 column (2.1 x 150 mm, 2.6 µm)
- Solvent A: 60% CAN/40% H2O containing 10 mM Ammonium formate and 0.1% DFA
- Solvent B: 90% IPA/10% ACN containing 10 mM Ammonium formate and 0.1% DFA
- Flow rate: 350ul/min
- Run time: 22min

Orbitrap Exploris™ 120 MS

- dd MS/MS experiment set up
- Full MS (+), 120K at m/z 200
- dd MS/MS, top 3, 30K at m/z 200



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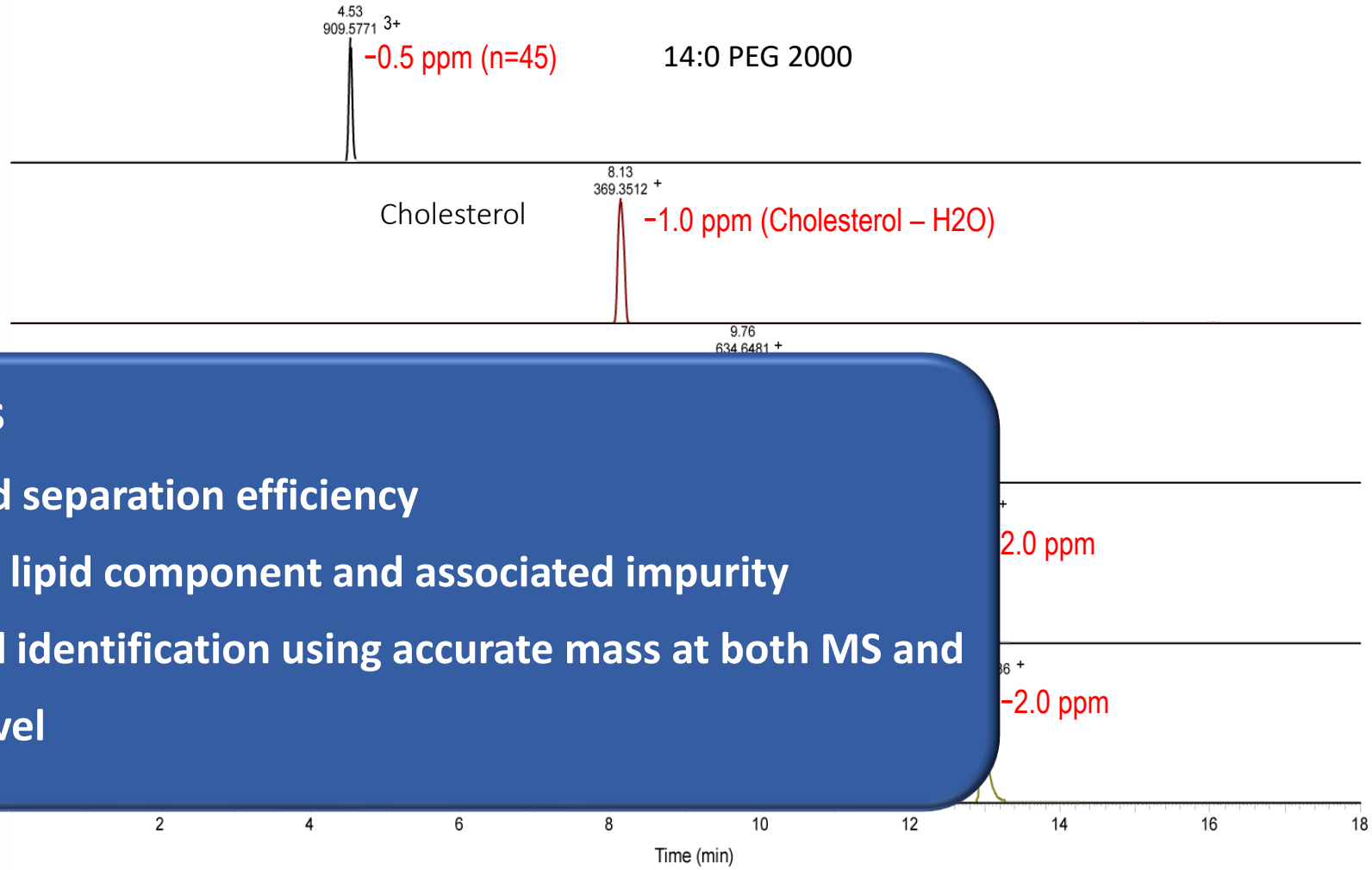
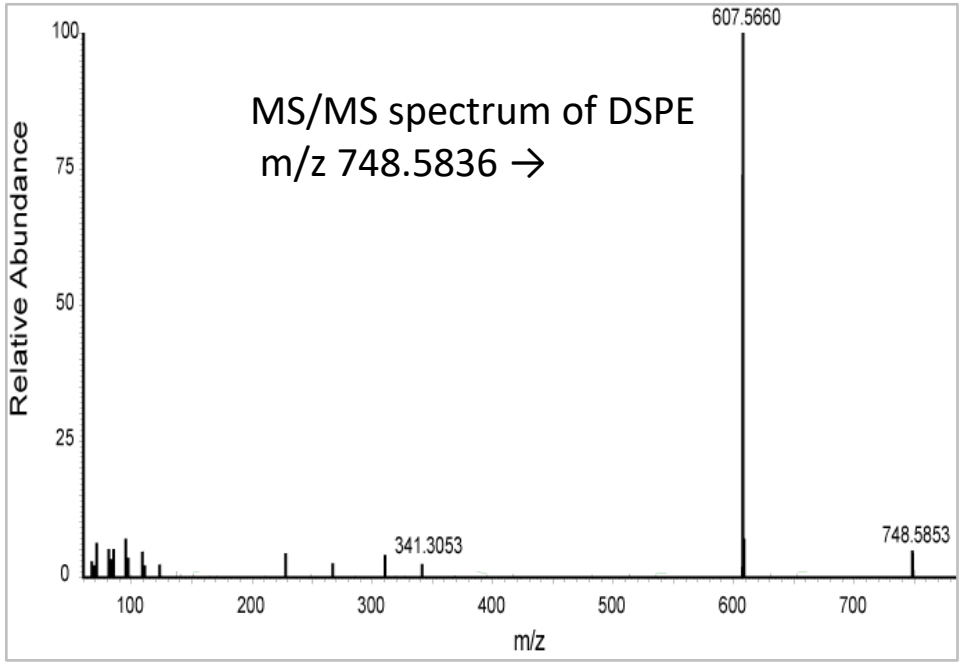


Advantages

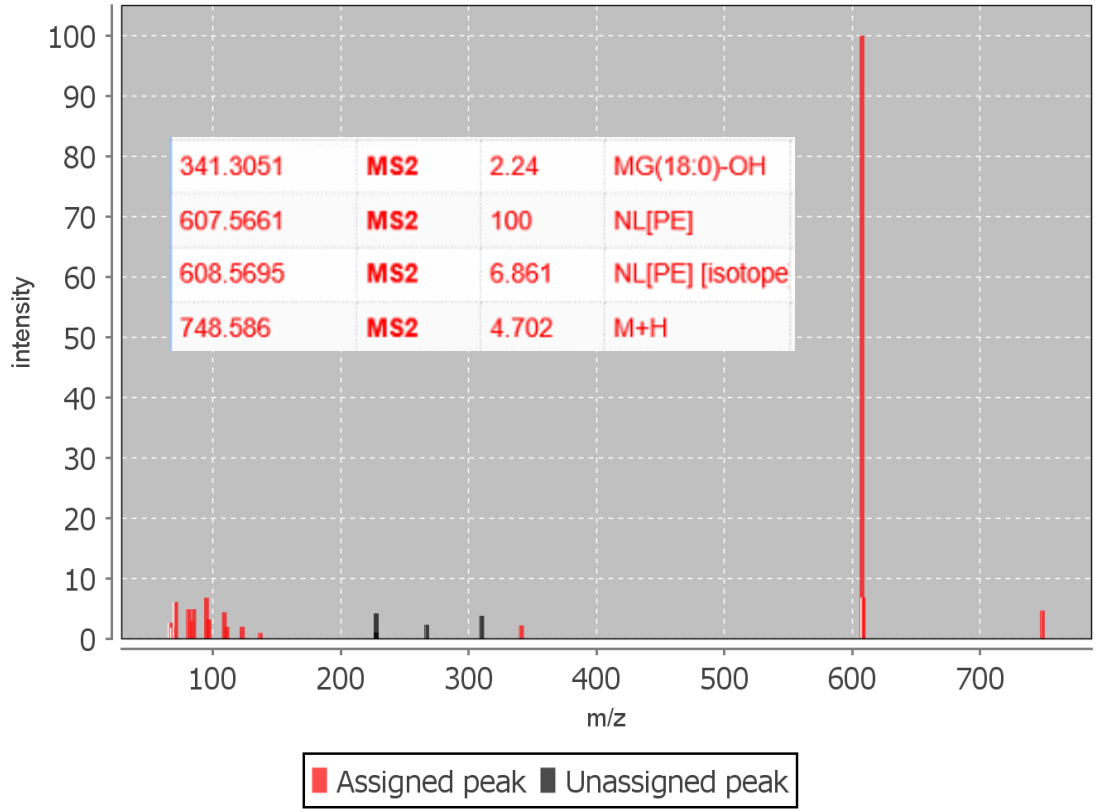
- **Great lipid separation efficiency**
- **Confident lipid component and associated impurity compound identification using accurate mass at both MS and MS/MS level**

Confident lipid structure confirmation using MS/MS



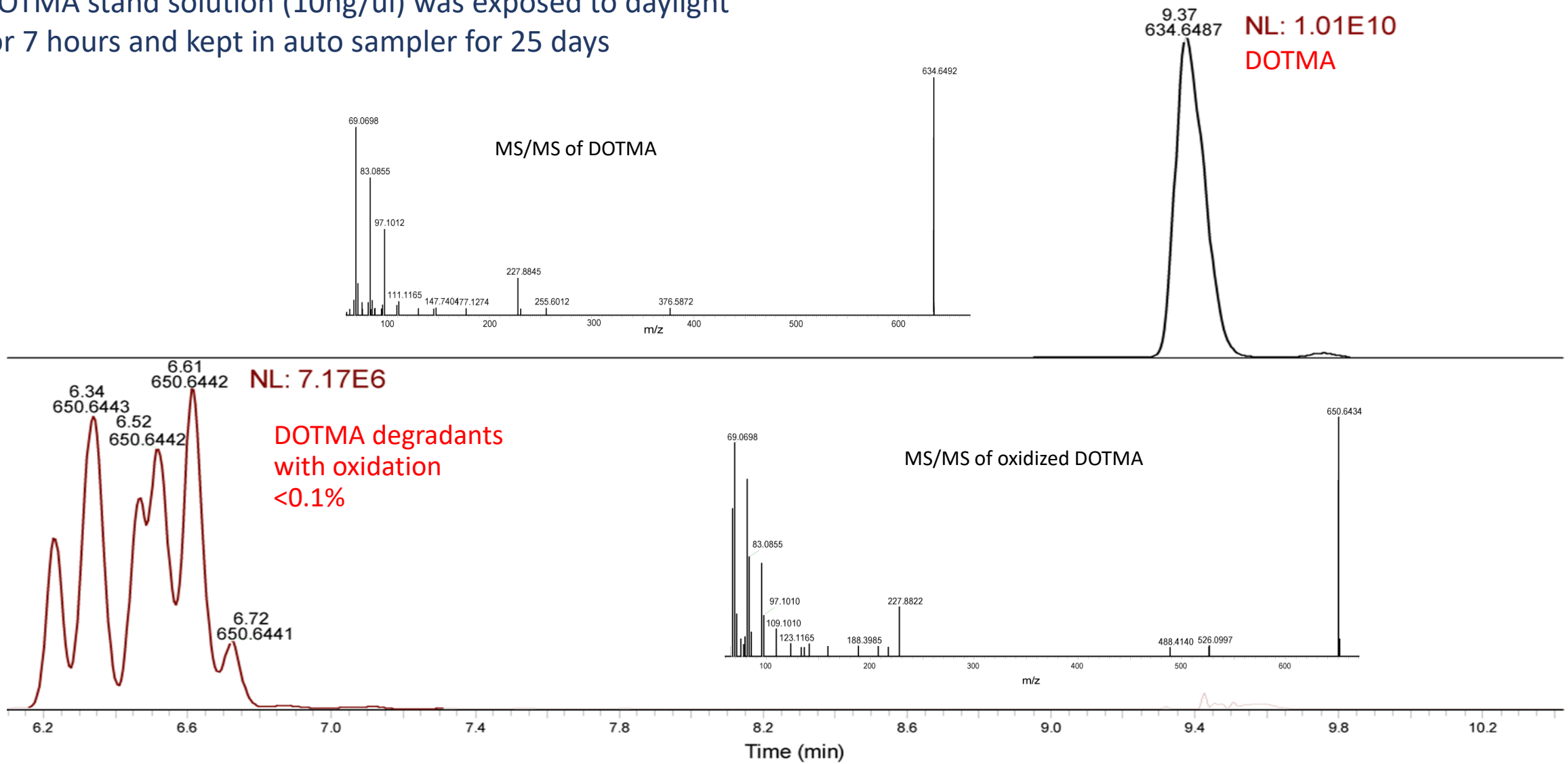
Search lipid database for lipid molecular identification

MS/MS data confirmed 18:0 fatty side chains of DSPE



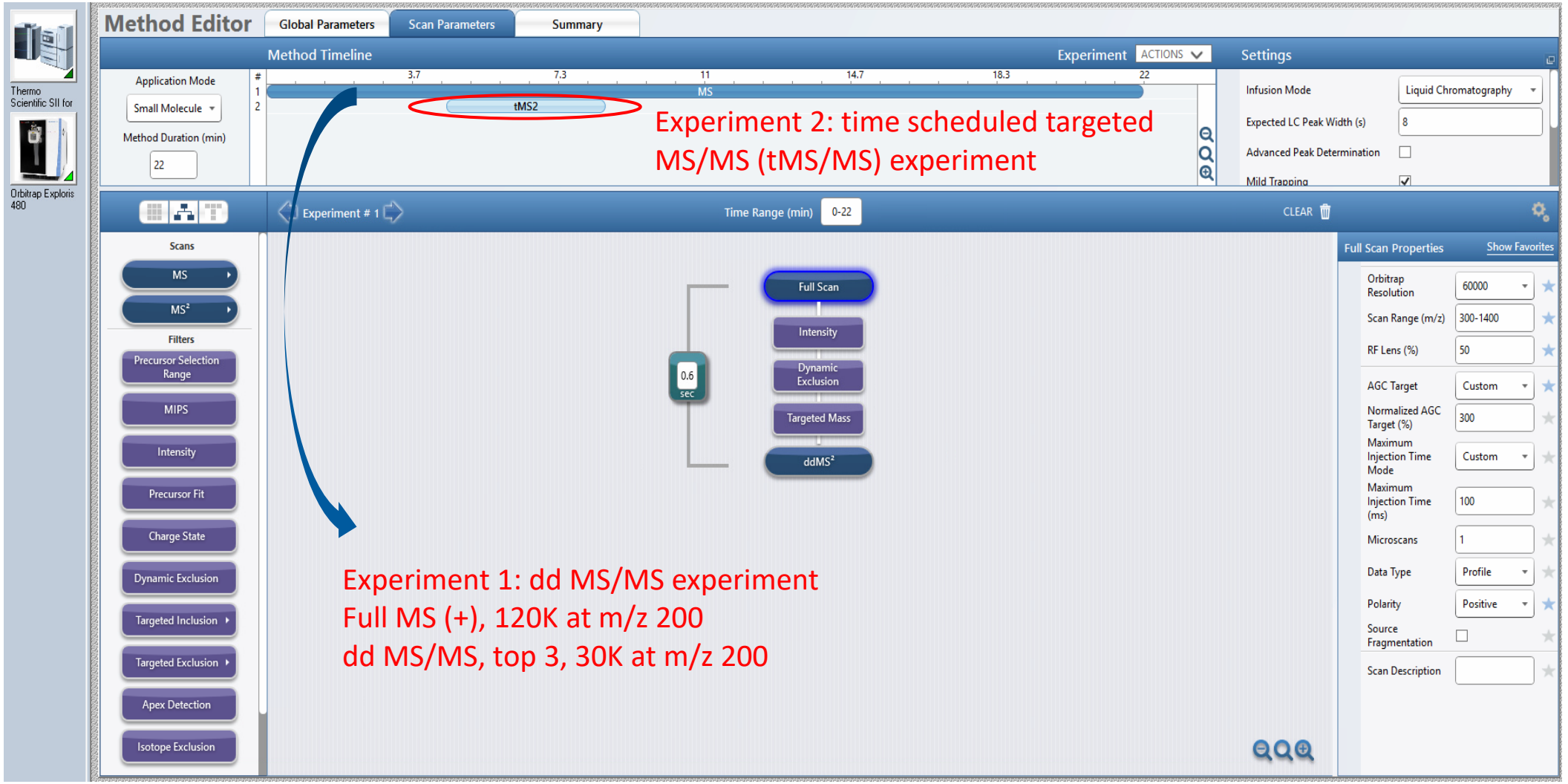
Rapid lipid degradant identification and relative quantification using LC MS-MS/MS

DOTMA stand solution (10ng/ul) was exposed to daylight for 7 hours and kept in auto sampler for 25 days



Simultaneous *in vivo* lipid metabolite analysis and targeted lipid quantification using LC MS-MS/MS

Two alternate experiments in a single LC-MS run



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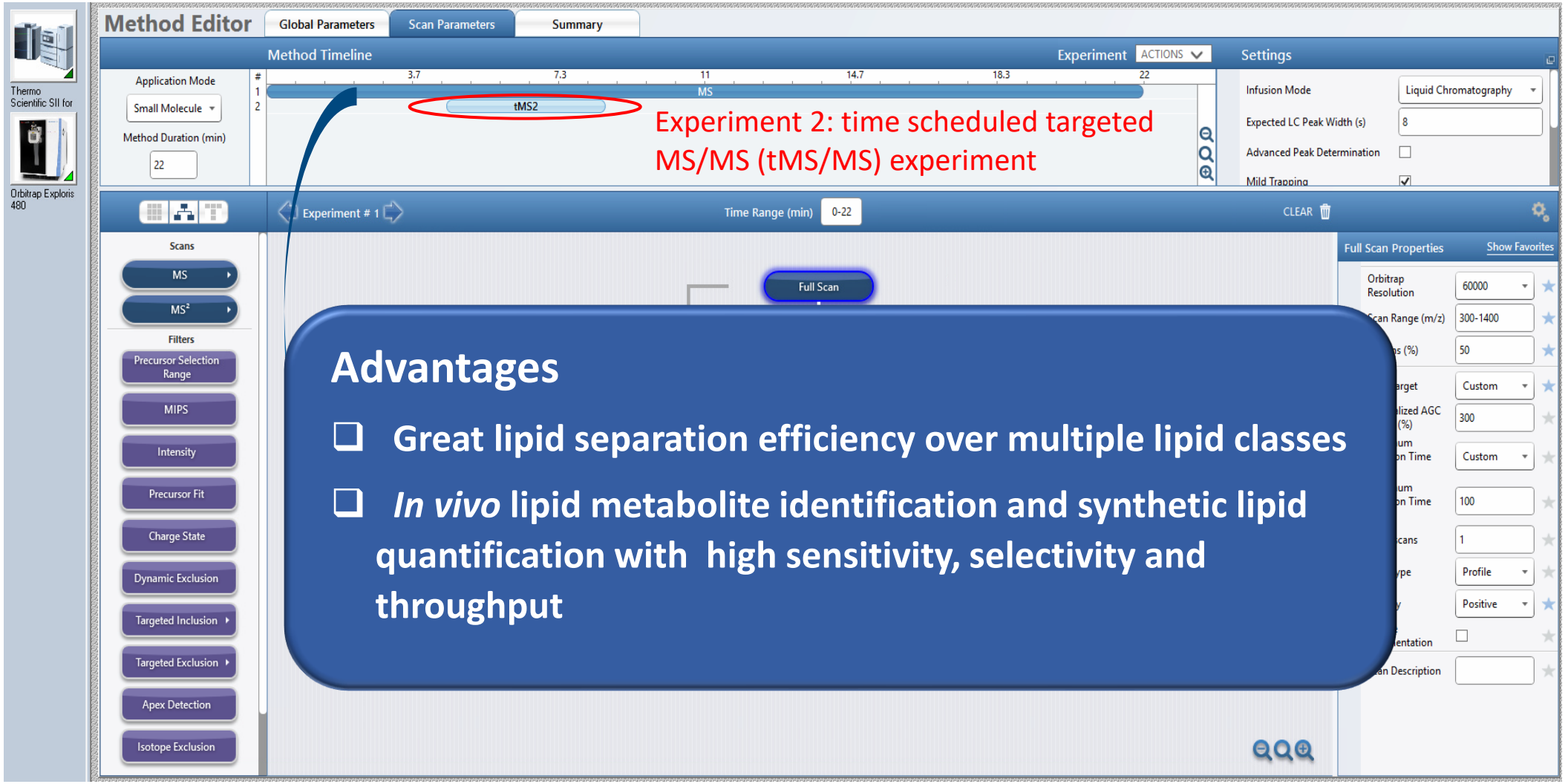
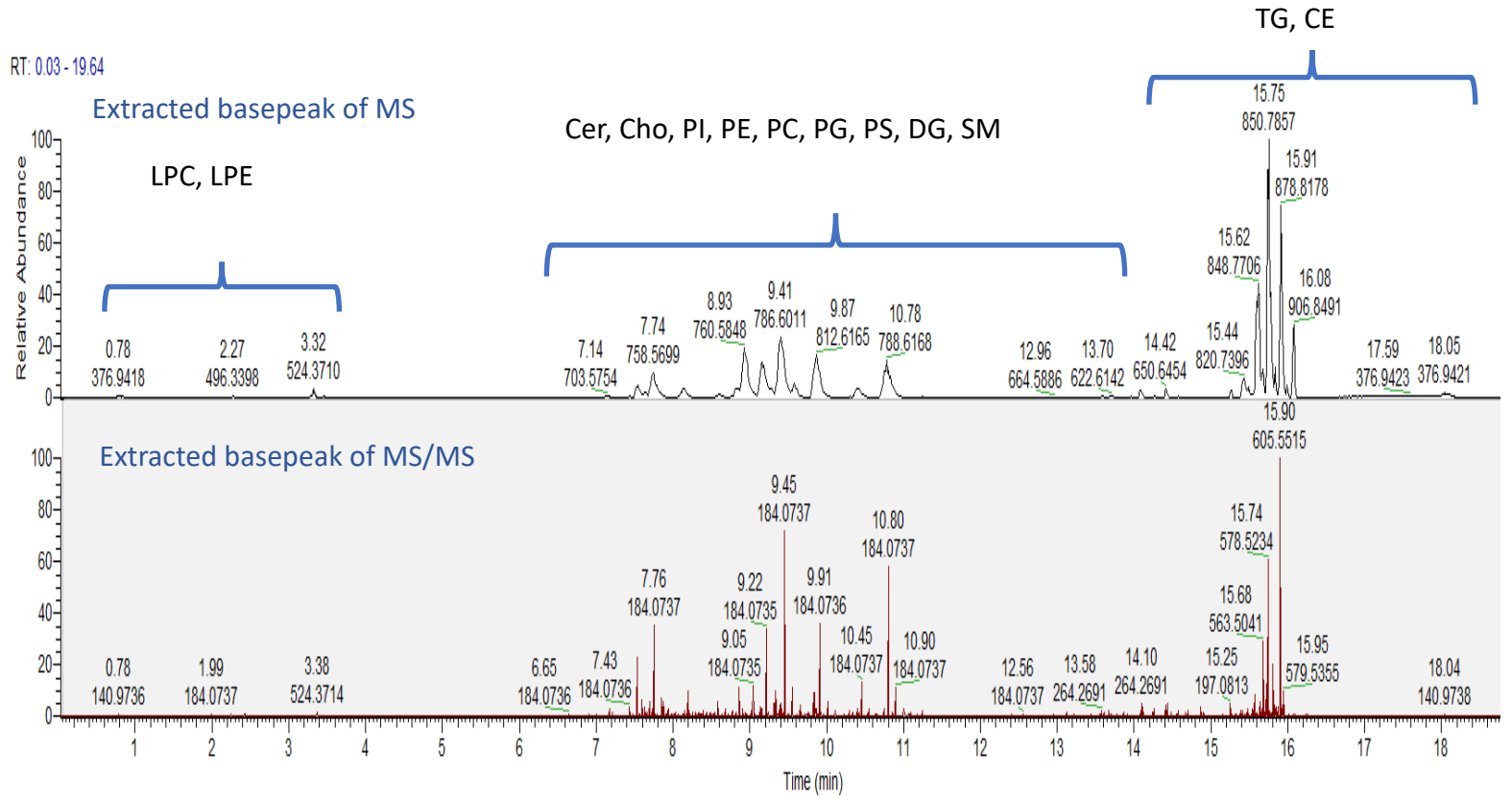


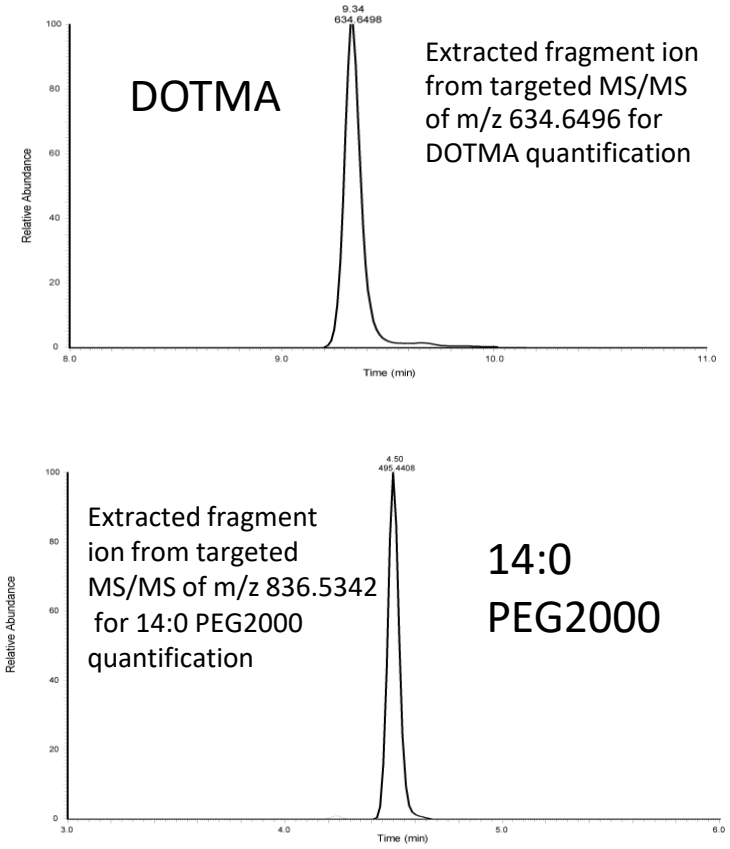
Applying the two alternate experiments method to a mimic biological matrix dilution series samples

Five lipid standards were spiked in the bovine liver total lipid extract as a dilution series at nine concentrations: 0.1 ng/mL, 0.25 ng/mL, 0.5 ng/mL, 1 ng/mL, 5 ng/mL, 10 ng/mL, 50 ng/mL, 100 ng/mL.

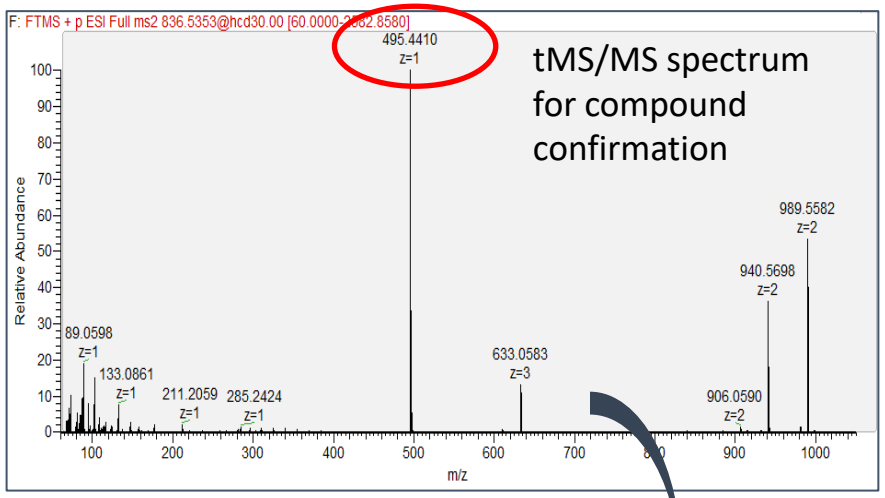
dd MS/MS for lipid metabolite identification and relative quantification



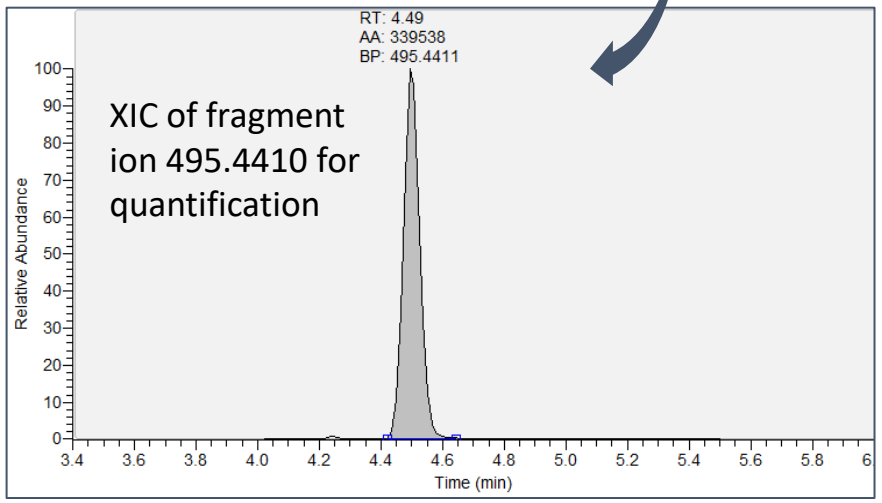
targeted MS/MS for highly sensitive and selective lipid quantification



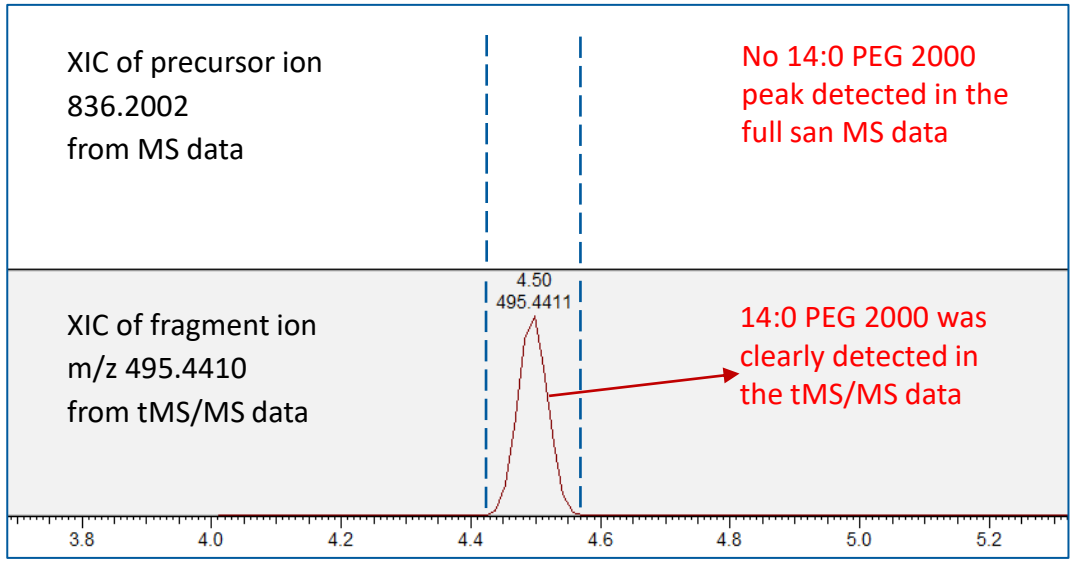
tMS/MS provides unique benefits for quantification



Most abundant and signature fragment ion (m/z 495.4410) selected for quantification

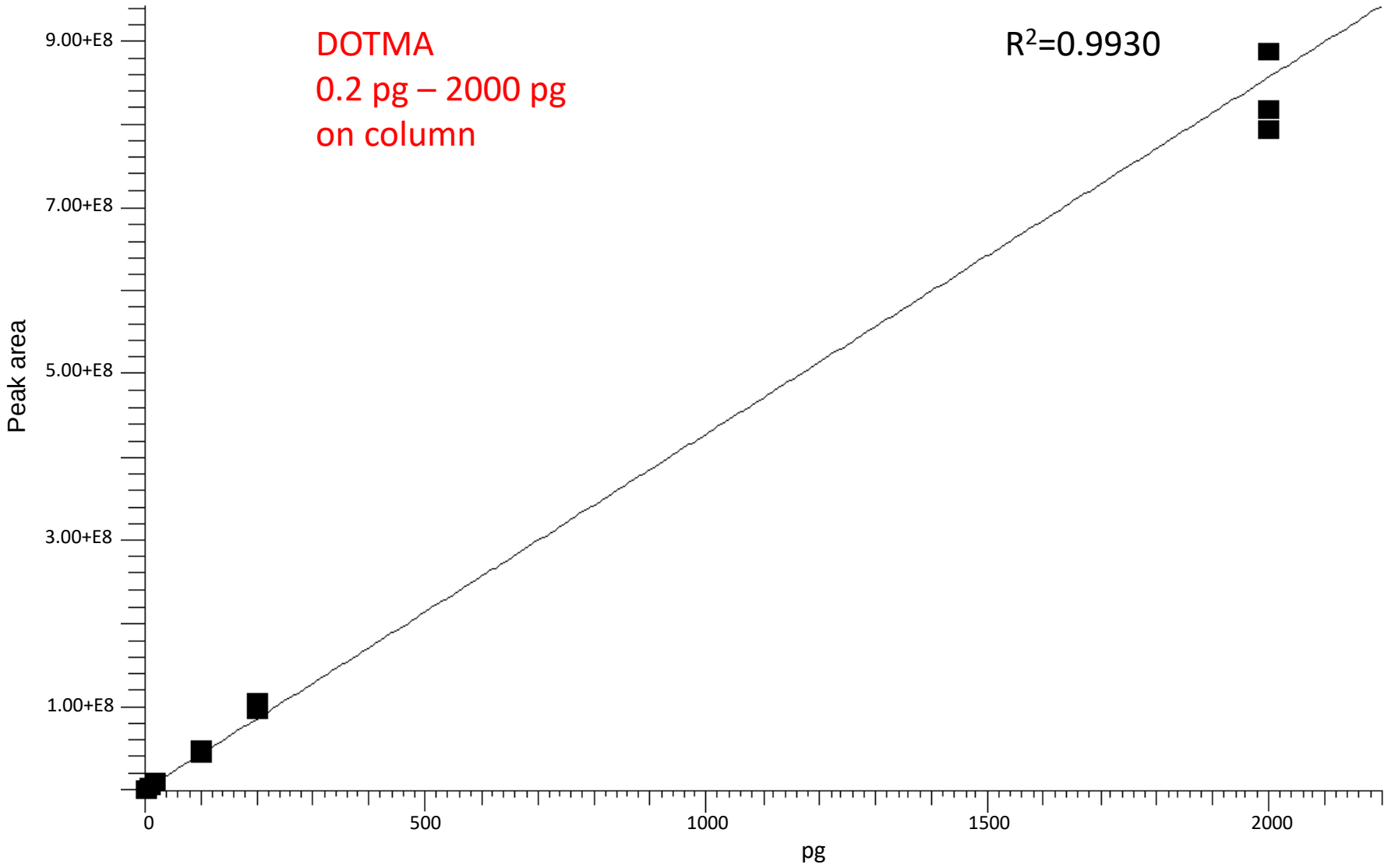


14:0 PEG 2000 spiked in the bovine liver extract at 0.25 pg/ µL



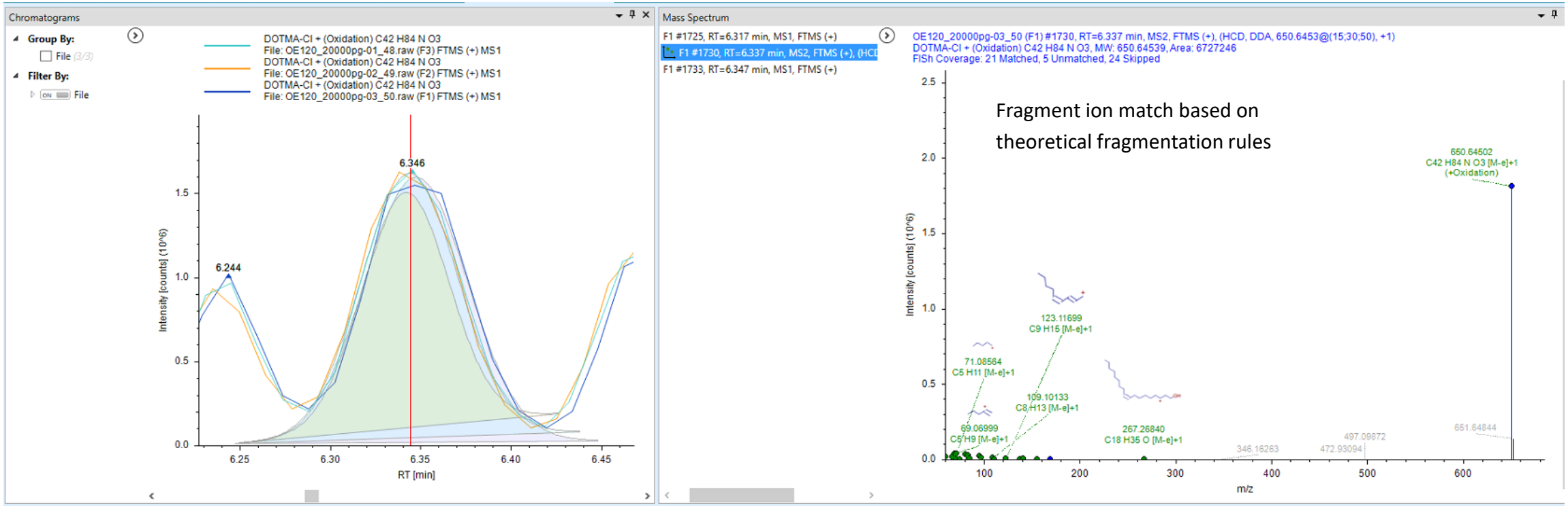
- Simultaneous compound identification and quantification
- Minimize the isomeric background noises for improving the LOD/LOQ by increased selectivity.
- Increase the ion signal for improving the LOD/LOQ by increased ion trapping efficiency

Calibration line for spiked-in DOTMA using tMS/MS data



DOTMA degradant characterization using dd MS/MS data

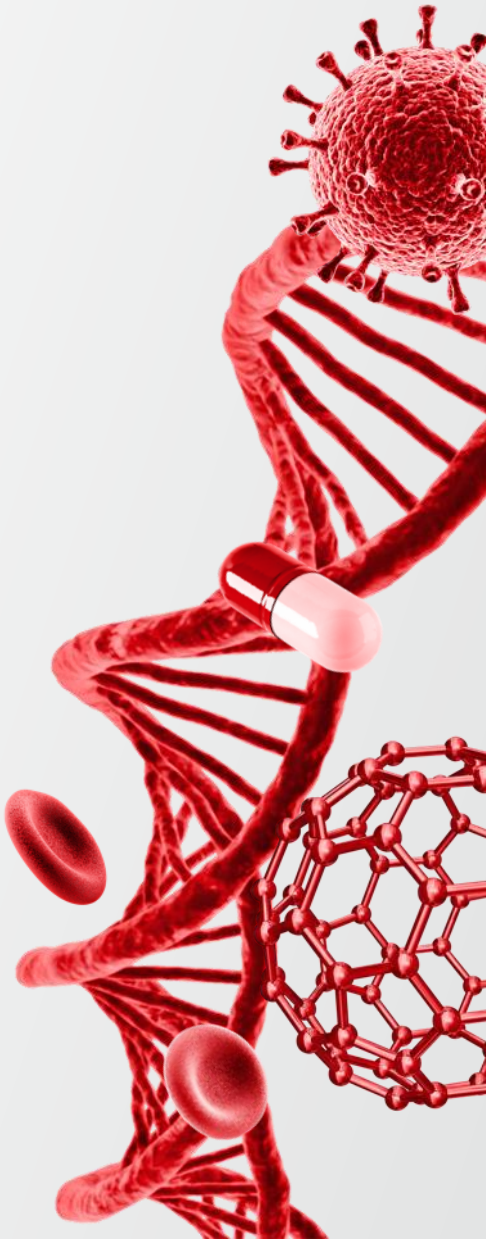
Compound Discoverer 3.3 software was used for DOTMA degradant identification



Expected Compounds | Expected Compounds per File | Expected Formulas | Merged Features | Expected Features | Input Files | Study Information

	Checked	Tags	Name	Formula	Parent Compound	Dealkylated	Transformations	Composition	Annot. ΔMass [ppm]	Calc. MW	RT [min]	FISh Coverage	Area (Max.)	MS2	Area		
1				C42 H84 N O2	DOTMA-Cl				-0.99	634.64958	9.349	87.78	19614503526		1.96e10	1.95e10	1.92e10
2				C42 H84 N O3	DOTMA-Cl		Oxidation	+(O)	0.42	650.64539	6.345	80.77	6727246		6.73e6	5.03e6	6.30e6
3				C42 H84 N O2	DOTMA-Cl				0.00	634.65021	9.728	78.82	138348307			1.38e8	
4				C42 H84 N O3	DOTMA-Cl		Oxidation	+(O)	0.54	650.64547	6.240	65.22	2804073		2.78e6	2.80e6	2.73e6
5				C42 H84 N O3	DOTMA-Cl		Oxidation	+(O)	0.67	650.64555	6.731	64.71	681842		6.82e5	5.38e5	5.91e5
6				C42 H84 N O3	DOTMA-Cl		Oxidation	+(O)	0.49	650.64544	6.630	60.00	1410800		1.41e6	7.91e5	1.13e6
7				C42 H84 N O3	DOTMA-Cl		Oxidation	+(O)	0.20	650.64525	6.472	57.89	4139022		4.14e6	2.89e6	3.73e6
8				C42 H84 N O4	DOTMA-Cl		Oxidation, Oxidation	+(O2)	0.52	666.64038	5.744	53.12	1345421		1.35e6	5.94e5	1.29e6
9				C42 H84 N O4	DOTMA-Cl		Oxidation, Oxidation	+(O2)	0.73	666.64052	5.634	52.24	796830		7.97e5	3.14e5	7.90e5

Case Study: SM-102 LNP Formulation (fLuc mRNA encapsulated)



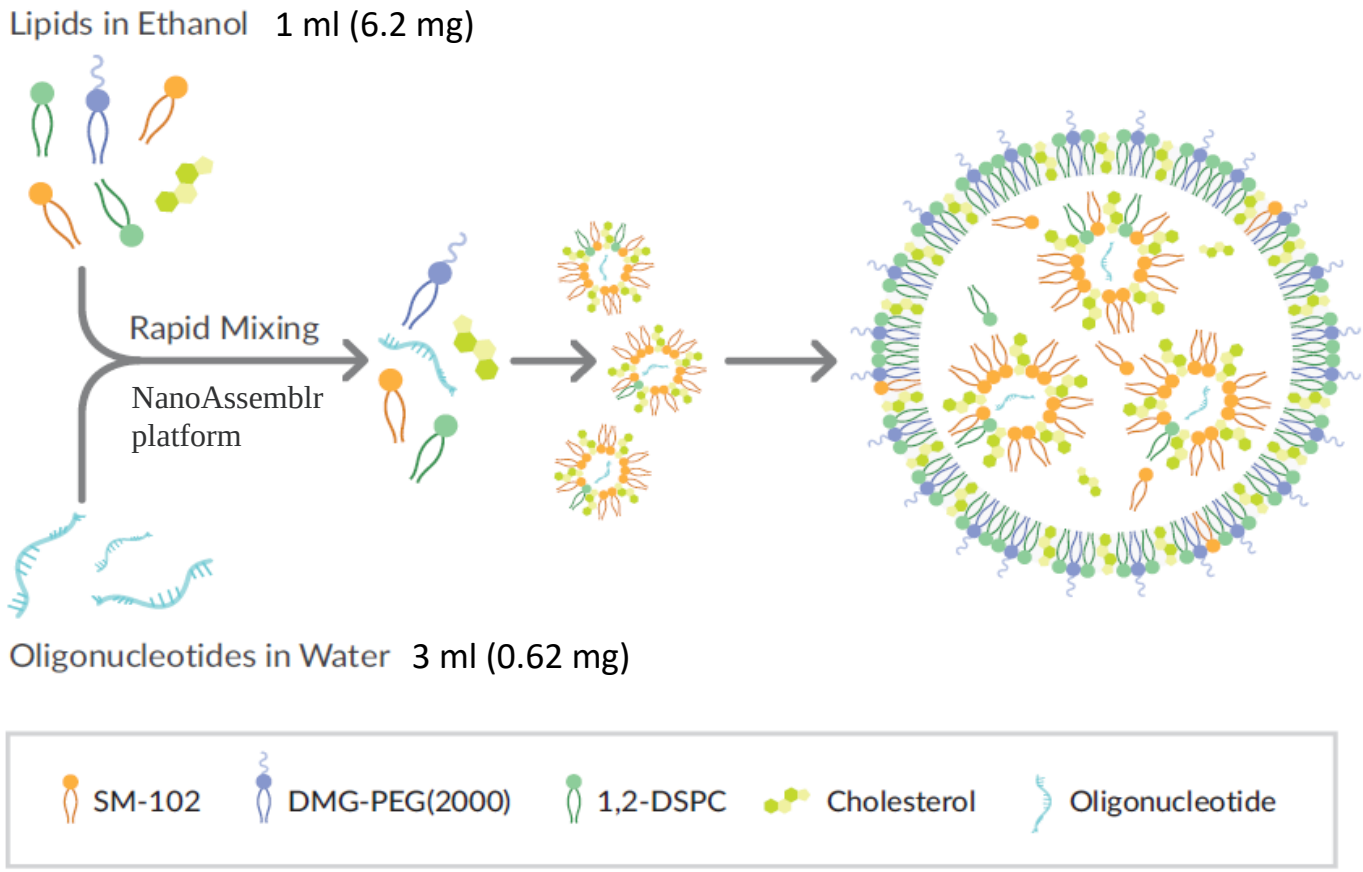
LNP formulation using LNP-102 Exploration Kit (Cayman)

Ethanolic Lipid Mixture

Lipid Mixture Component	MW	Molar Ratio	mg
SM-102	710.2	50	3.55
1,2-DSPC	790.2	10	0.79
Cholesterol	386.7	38.5	1.48
DMG-PEG(2000)	2,526	1.5	0.38
Total		100	6.2

Aqueous mRNA Solution

Add 0.62 mg fLuc mRNA (Trilink) to a separate tube and adjust the volume to 3.0 ml with 50 mM sodium acetate, pH 5.0.

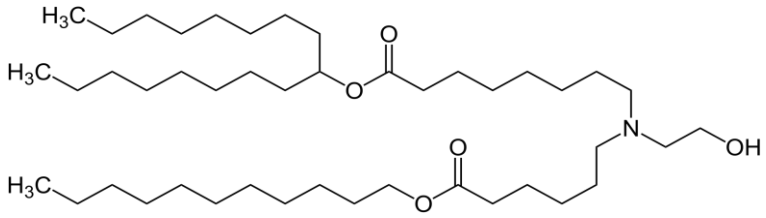


- The lipid mixture was combined with the acidification buffer of 25 mM sodium acetate (pH 5.0) containing mRNA at a volume ratio of 3:1 (mRNA:Lipids) using a microfluidic mixer.
- The formulation was dialyzed against PBS (pH 7.4) for 18 hours.

fLuc mRNA-formulated SM-102 LNP

Mice were injected IM with 100 µl containing 5 ug of firefly luciferase mRNA formulated in SM-102 LNPs

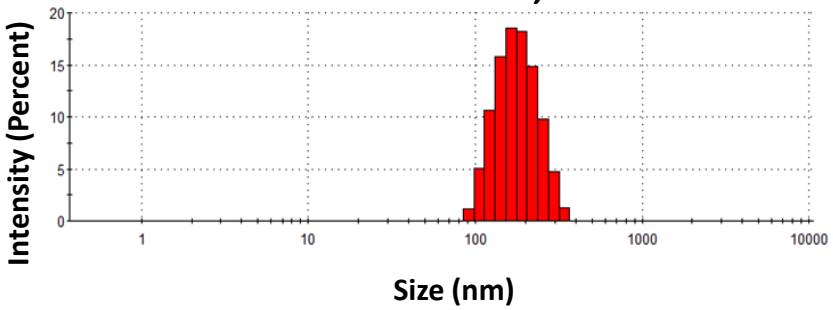
A The structure of lipid SM-102



B Dynamic light scattering analysis of LNP size, size distribution and mRNA encapsulation of fLuc mRNA-LNP

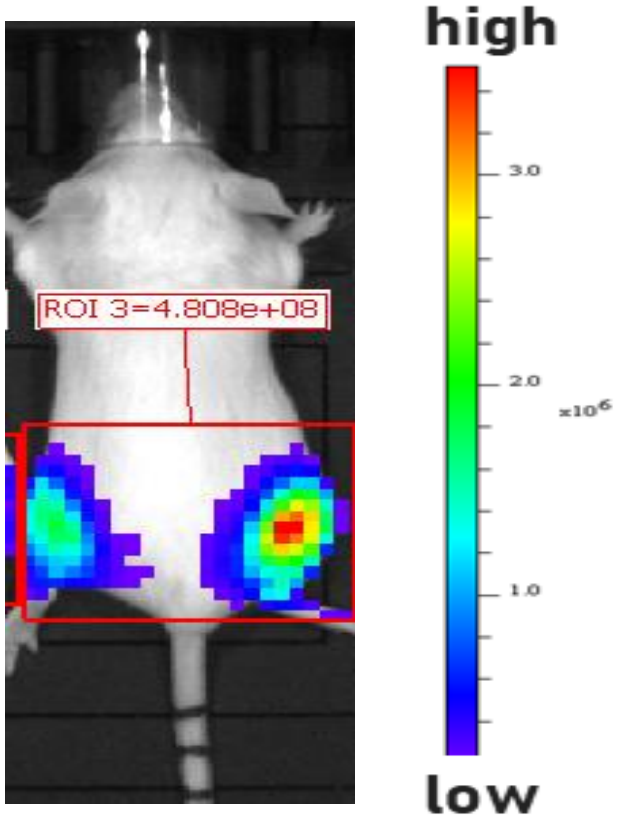
Encapsulation = 96.8%

SM-102 LNP: 169 nm, PDI 0.068



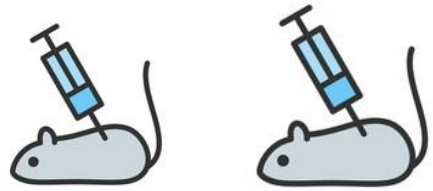
C *In vivo* bioluminescence images following IM injection of fLuc mRNA-LNP

SM-102



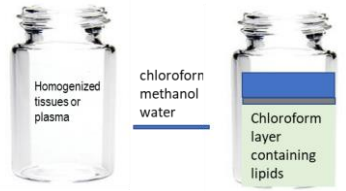
Sample preparation for SM-102 LNP lipid analysis

- At 1, 2, 4, 8, and 24 h post-injection, 2 mice were sacrificed, and the plasma, spleen, liver, and site of injection muscle were harvested.

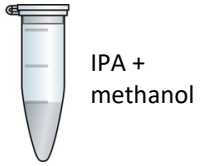


- Tissue samples were homogenized using tissue homogenizer with ceramic beads by following the addition of 19 equivalents (w/v) of high purified water.

- Lipid extraction and protein precipitation were carried out by adding methanol and chloroform solvents.



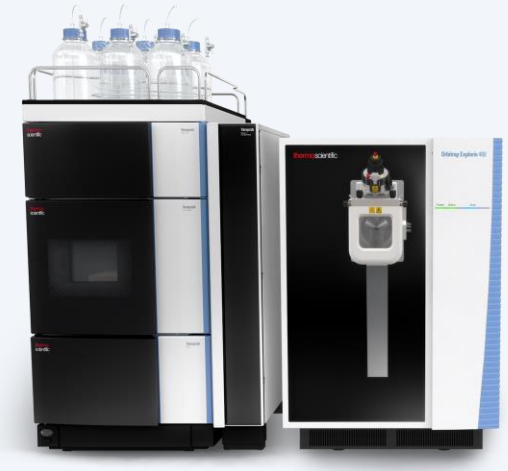
- The chloroform layer containing lipids was dried down and reconstitute in IPA/methanol/ (1:1).



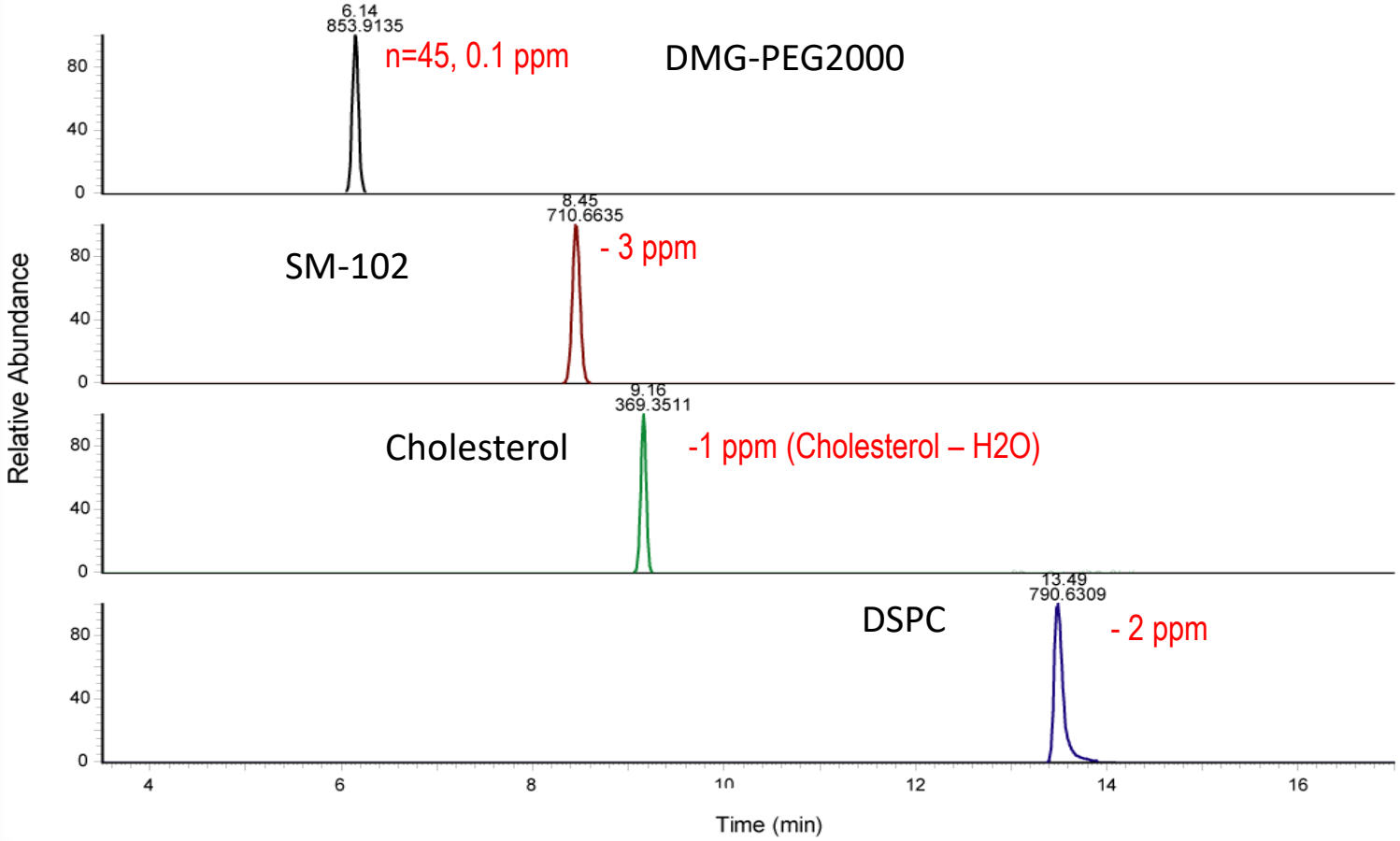
SM-102 LNP lipid analysis using the LC MS-MS/MS method

Vanquish H system
- Thermo Scientific™ Accucore™ C30 column (2.1 x 150 mm, 2.6 µm)

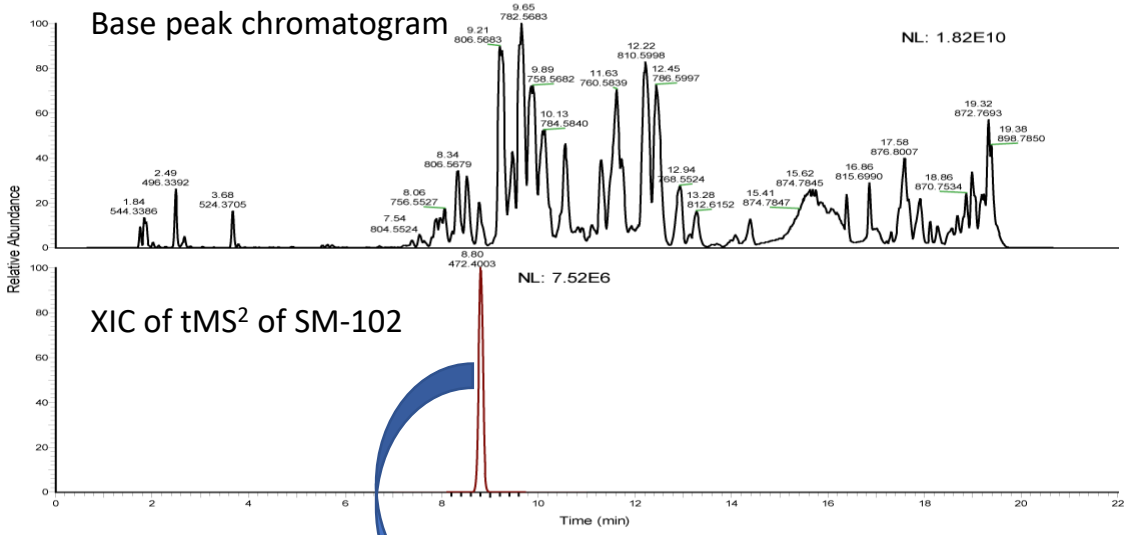
Orbitrap Exploris™ 480 MS
- dd MS/MS experiment followed by the time scheduled targeted MS/MS experiment

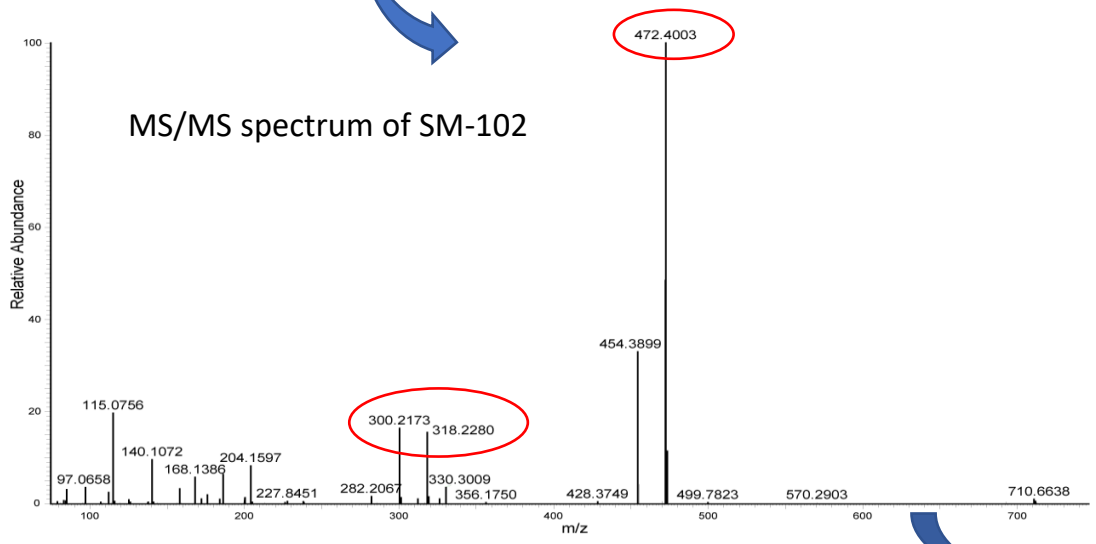


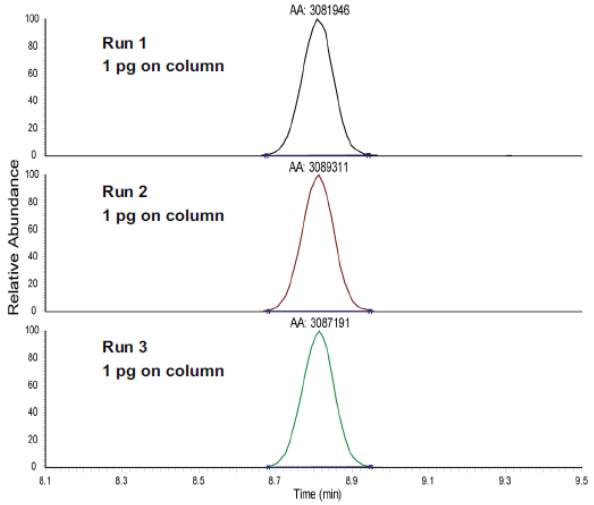
Lipid standards spiked in the control mice liver samples



Calibration curve for SM-102 spiked in the control mice liver samples

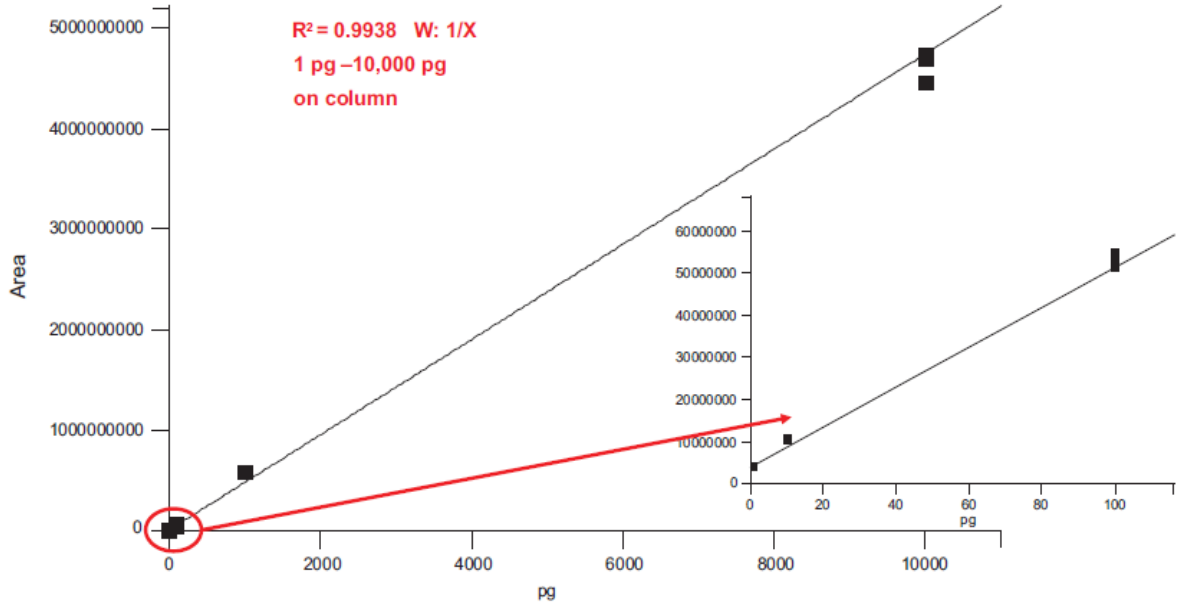






Extracted fragment ion chromatogram (300.2173 + 318.2280 + 472.4003)

CV% (n=3) of peak area: <5%

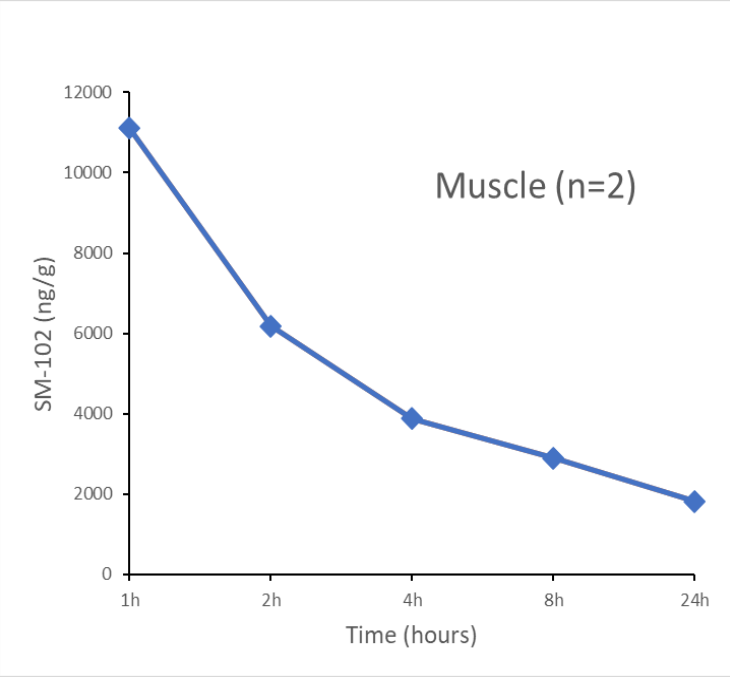


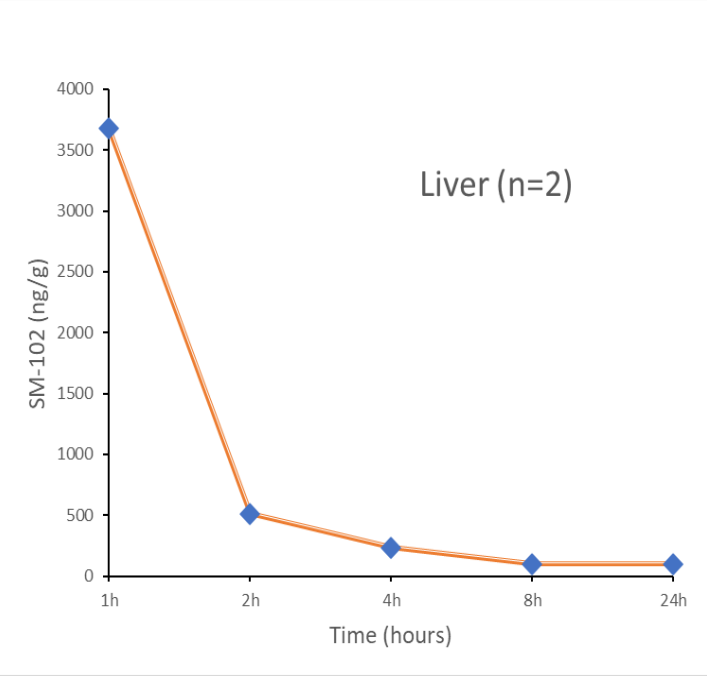
SM-102 clearance rates observed from mice organs

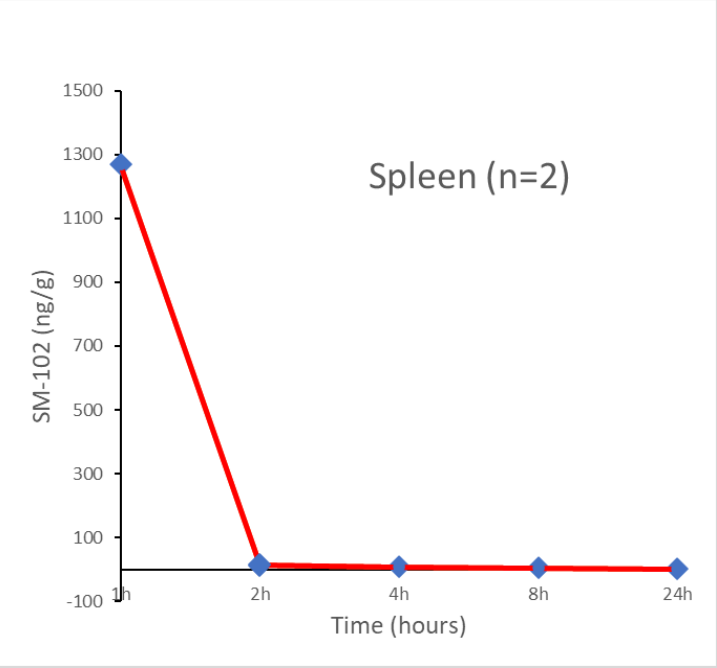
Sample #	Mouse	Sample weight (g)	Lipid extract in IPA/MeOH (µl), 0.5mg/µl	2 µl injcetion, observed amount (pg)
4	1 h -1	0.05	100	12860
8	1 h -2	0.04	80	9360
12	2 h -1	0.04	80	5452
16	2 h -2	0.11	220	7099
20	4 h -1	0.06	120	3513
24	4 h -2	0.06	120	4240
28	8 h -1	0.1	200	2592
32	8 h -2	0.11	220	3194
36	24 h -1	0.05	100	1437
40	24 h -2	0.06	120	2135

Sample #	Mouse	Sample weight (g)	Lipid extract in IPA/MeOH (µl), 0.5mg/µl	2 µl injcetion, observed amount (pg)
2	1 h -1	0.05	100	3119
6	1 h -2	0.05	100	4235
10	2 h -1	0.06	120	398
14	2 h -2	0.05	100	625
18	4 h -1	0.06	120	228
22	4 h -2	0.04	80	242
26	8 h -1	0.05	100	115
30	8 h -2	0.1	200	83
34	24 h -1	0.05	100	102
38	24 h -2	0.05	100	91

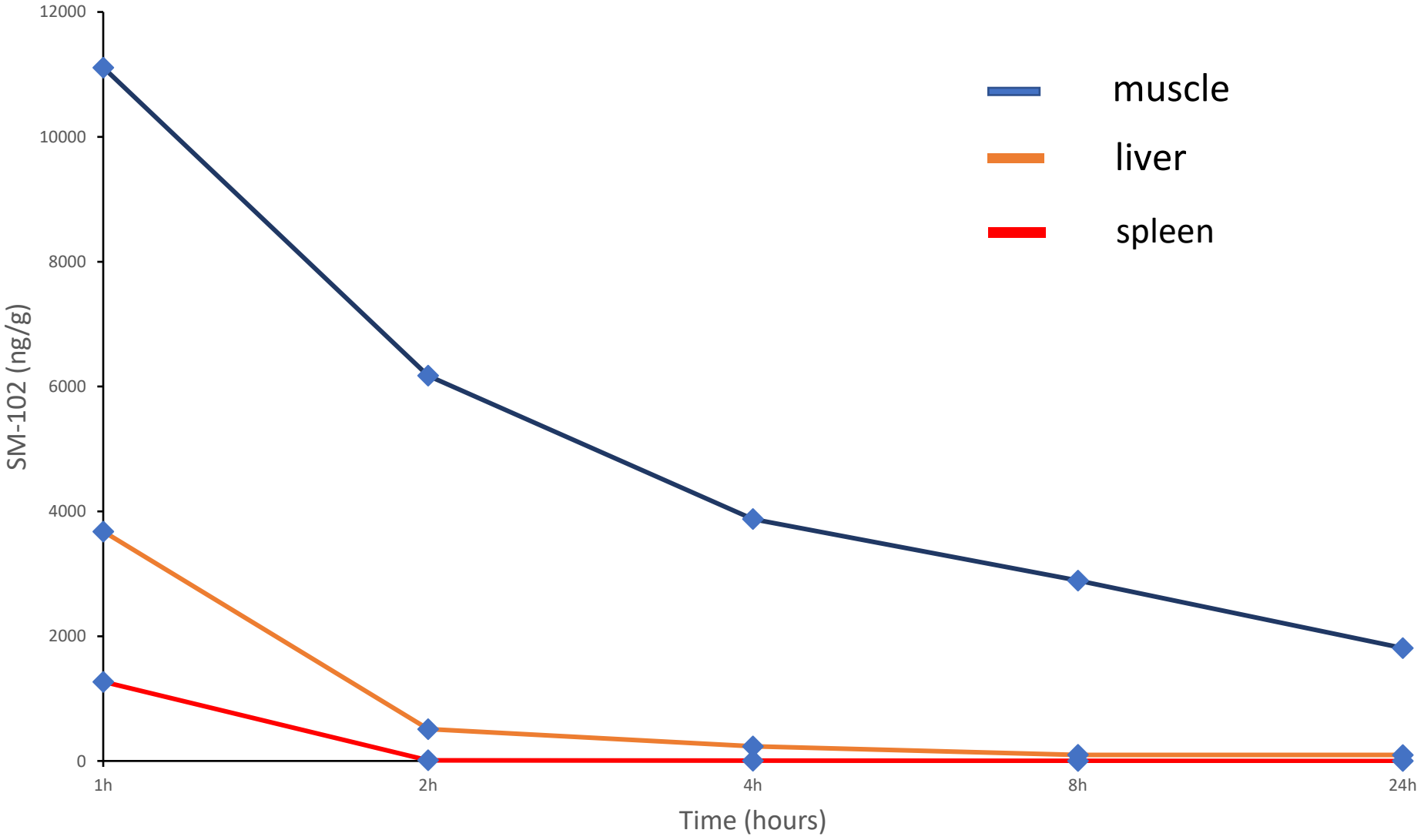
Sample #	Mouse	Sample weight (g)	Lipid extract in IPA/MeOH (µl), 0.5mg/µl	2 µl injcetion, observed amount (pg)
3	1 h -1	0.04	80	1614
7	1 h -2	0.06	120	927
11	2 h -1	0.06	120	8
15	2 h -2	0.05	80	17
19	4 h -1	0.06	120	6
23	4 h -2	0.06	120	9
27	8 h -1	0.1	200	4
31	8 h -2	0.1	200	3
35	24 h -1	0.1	200	<1
39	24 h -2	0.06	120	<1



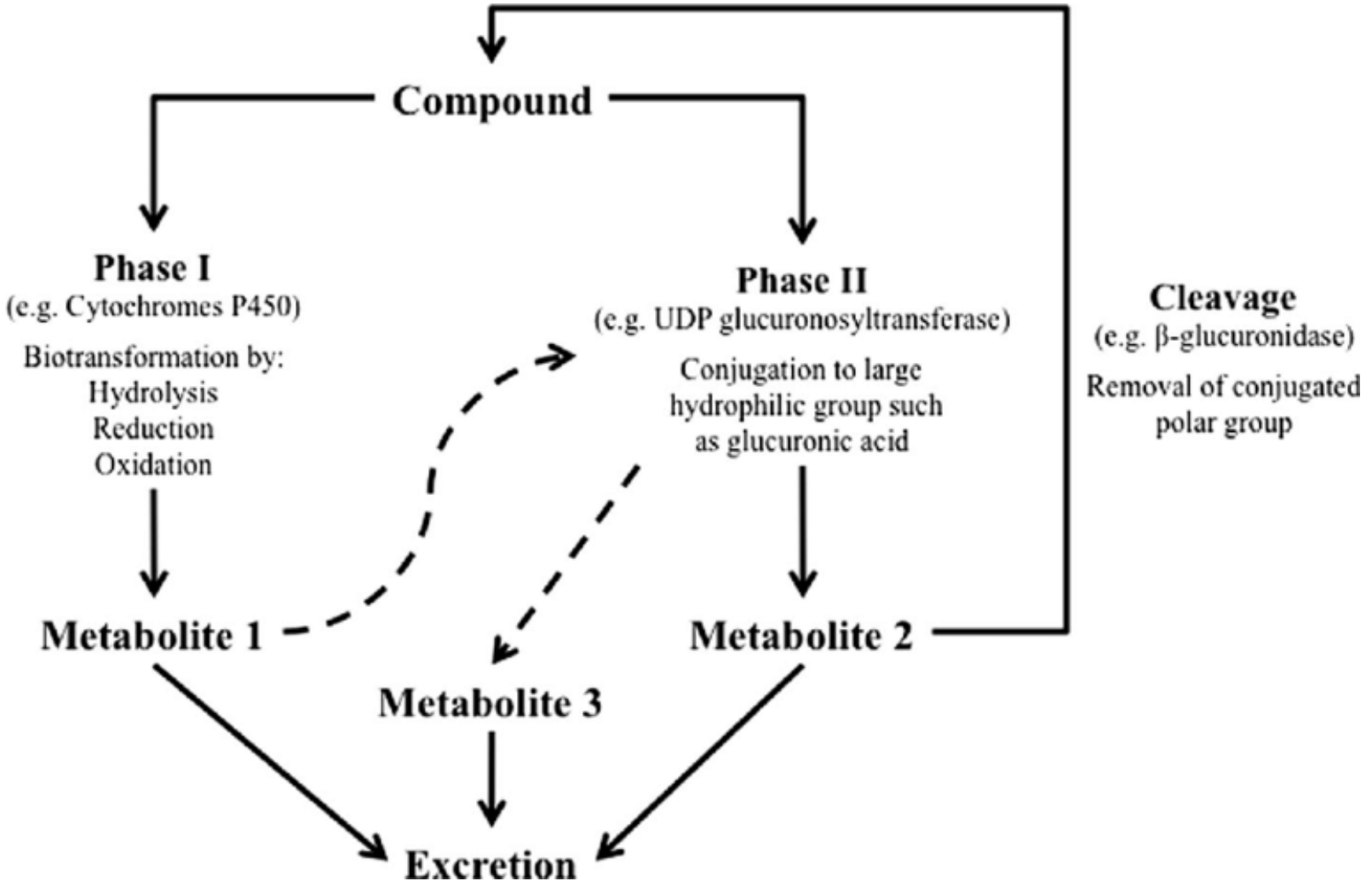




SM-102 clearance rate comparison across different mice organs



Synthetic SM-102 metabolism in vivo



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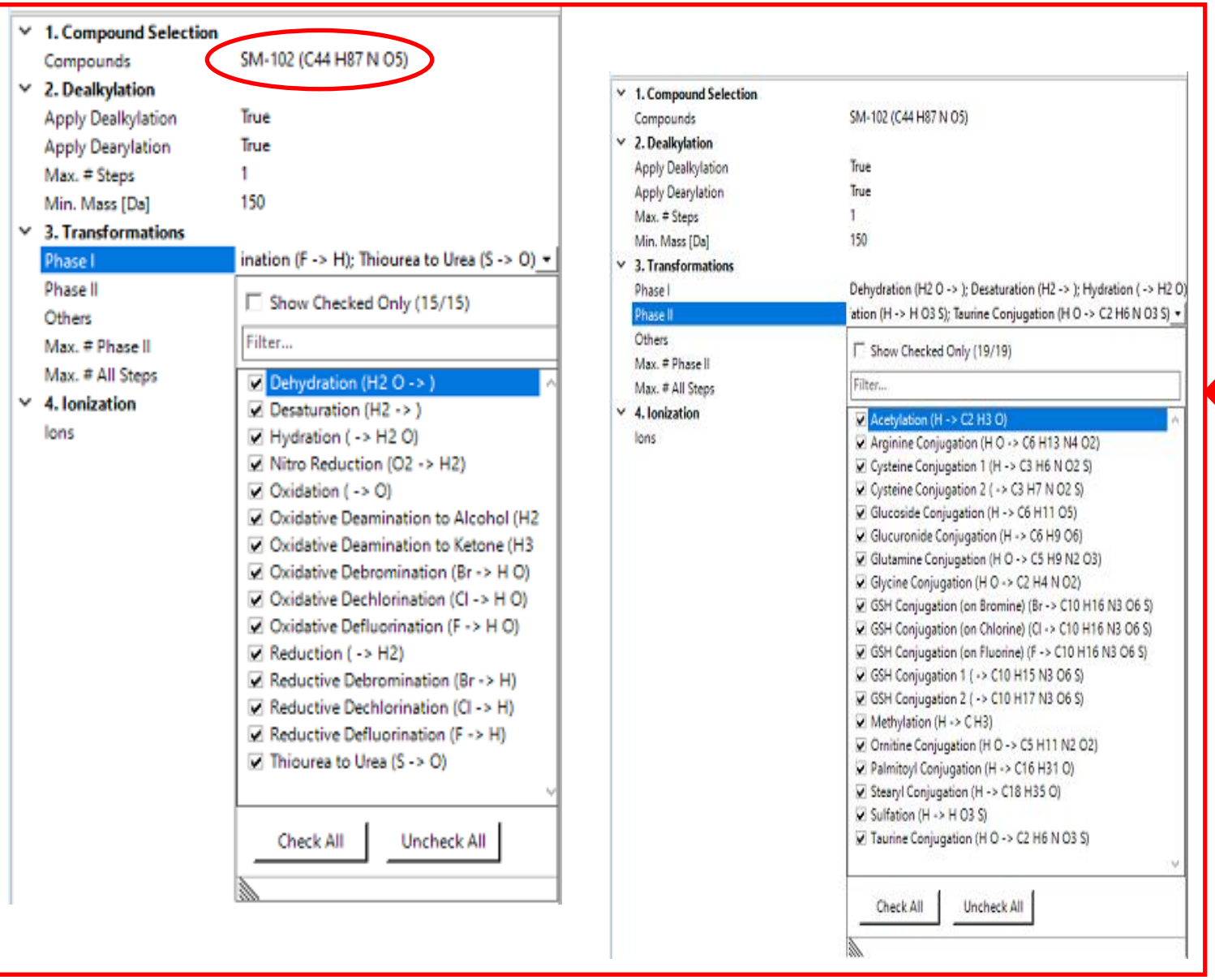
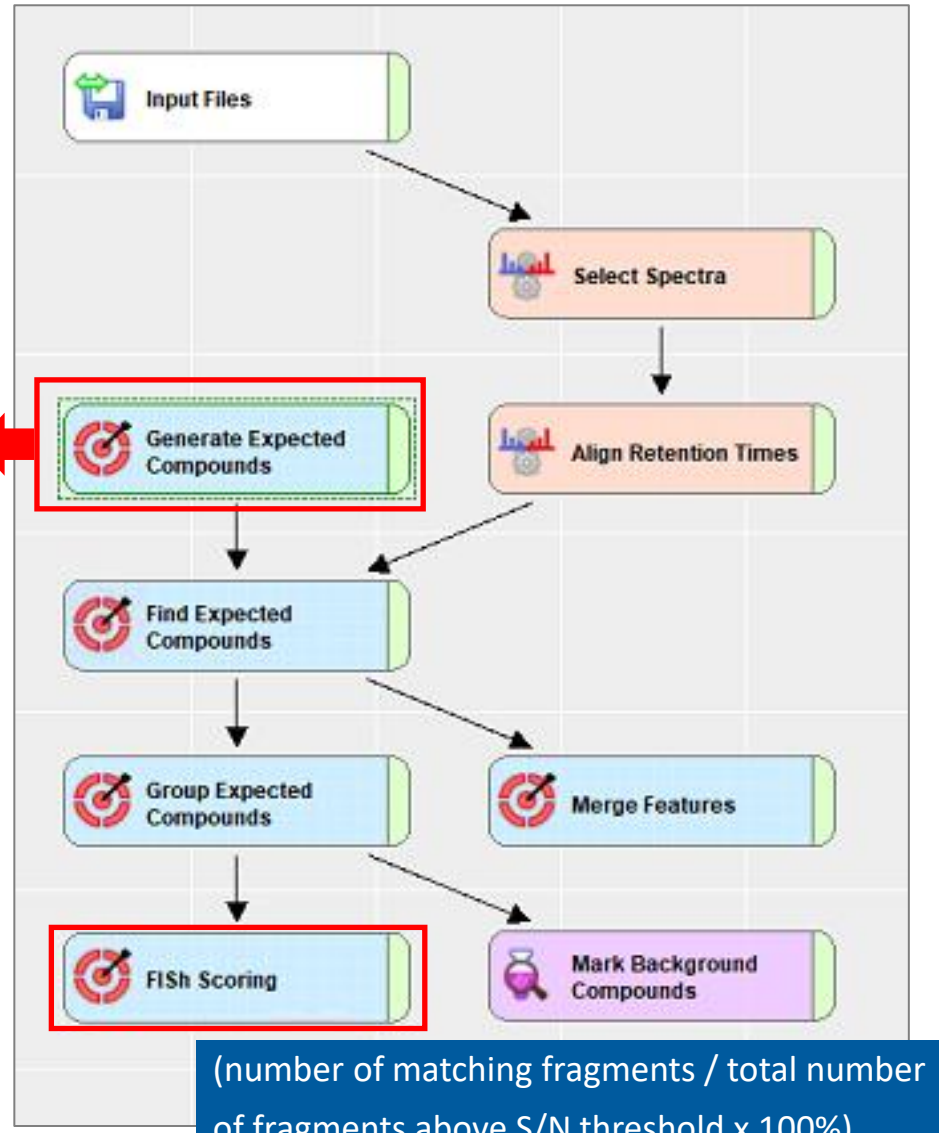
SM-102 potential metabolite characterization using the Compound Discoverer 3.3 software

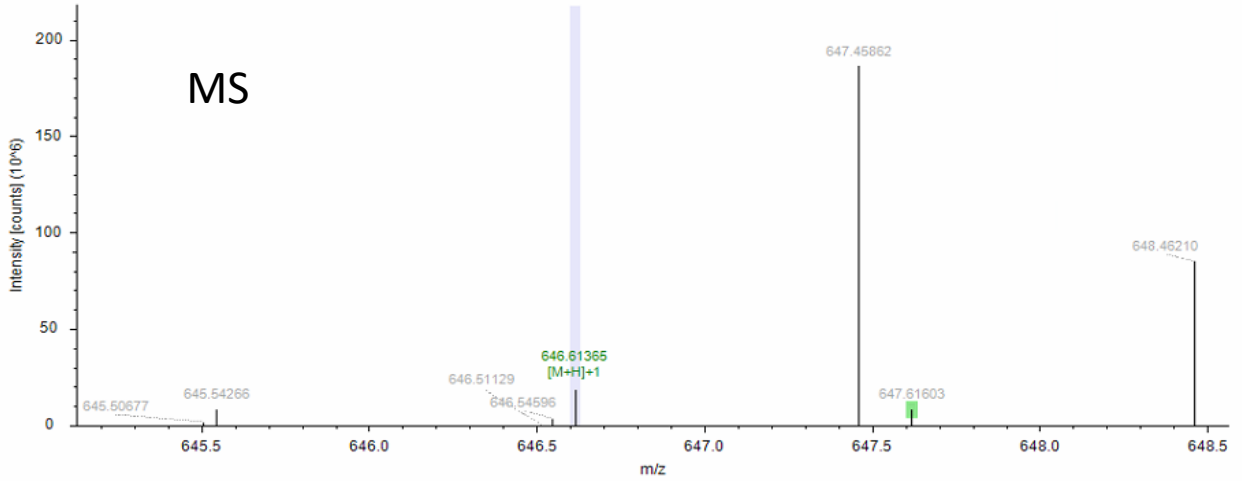


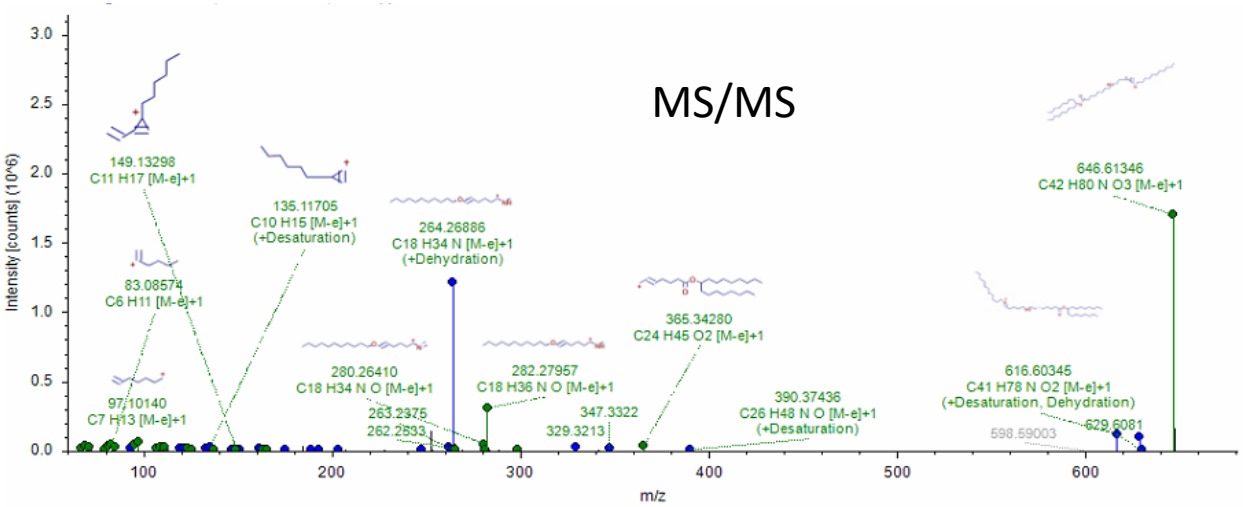


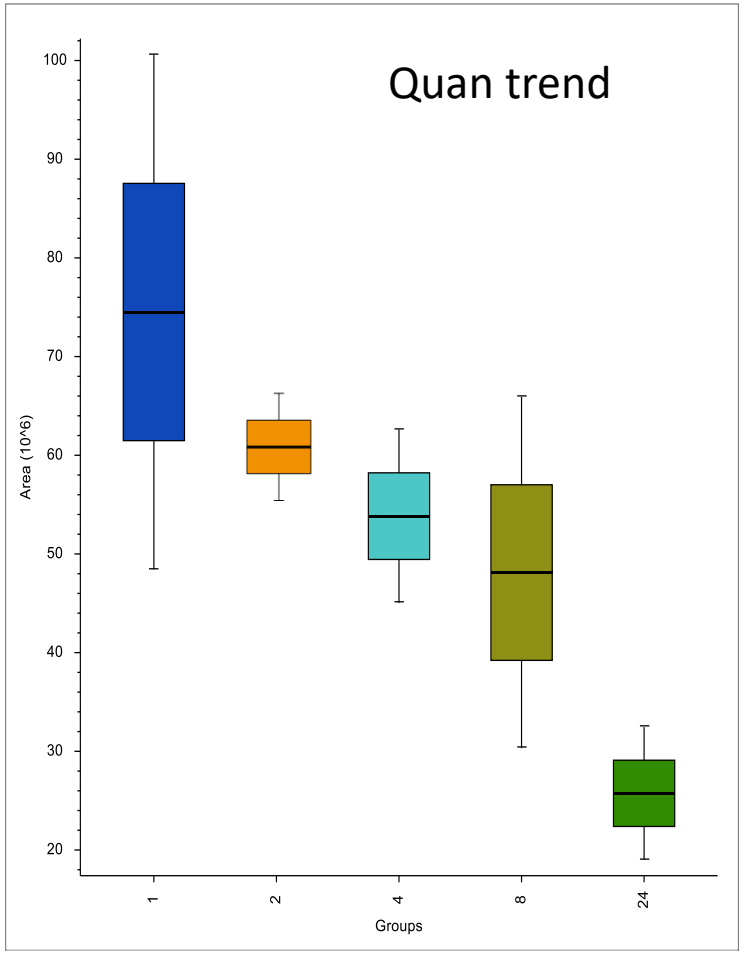
(number of matching fragments / total number of fragments above S/N threshold x 100%)

Potential metabolite of SM-102 from the liver samples

Formula	Parent Compound	Dealkylated	Transformations	Composition Change	Annot. ΔMass [ppm]	Calc. MW	m/z	RT [min]	FISh Coverage	Area (Max.)	MS2	Reference Ion
C42 H79 N O3	SM-102	X	Dehydration, Desaturation	-(C2 H8 O2)	0.79	645.60651	646.61378	16.816	60.00	100584341		[M+H]+1

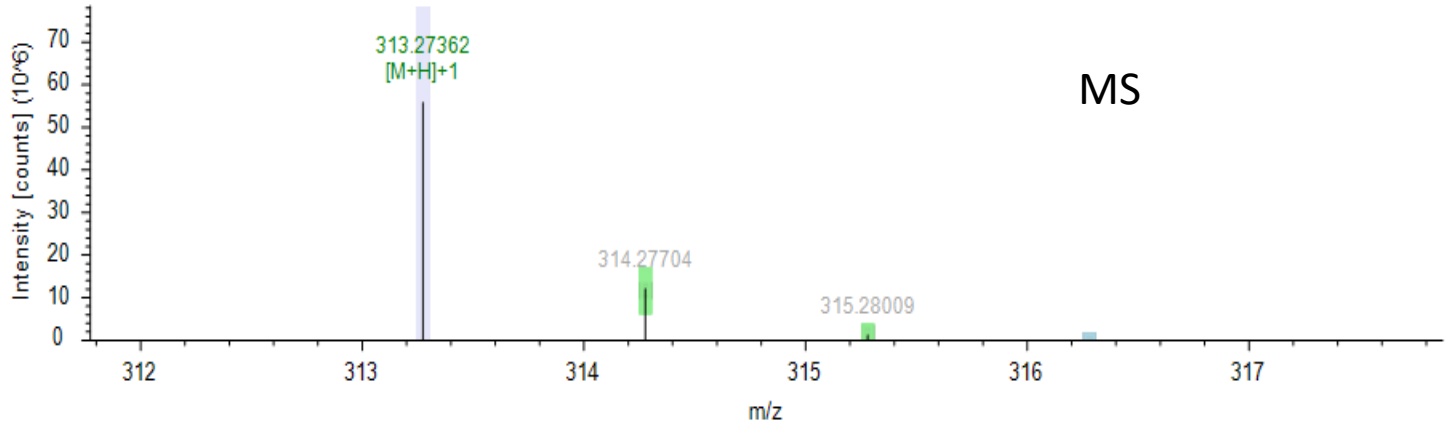


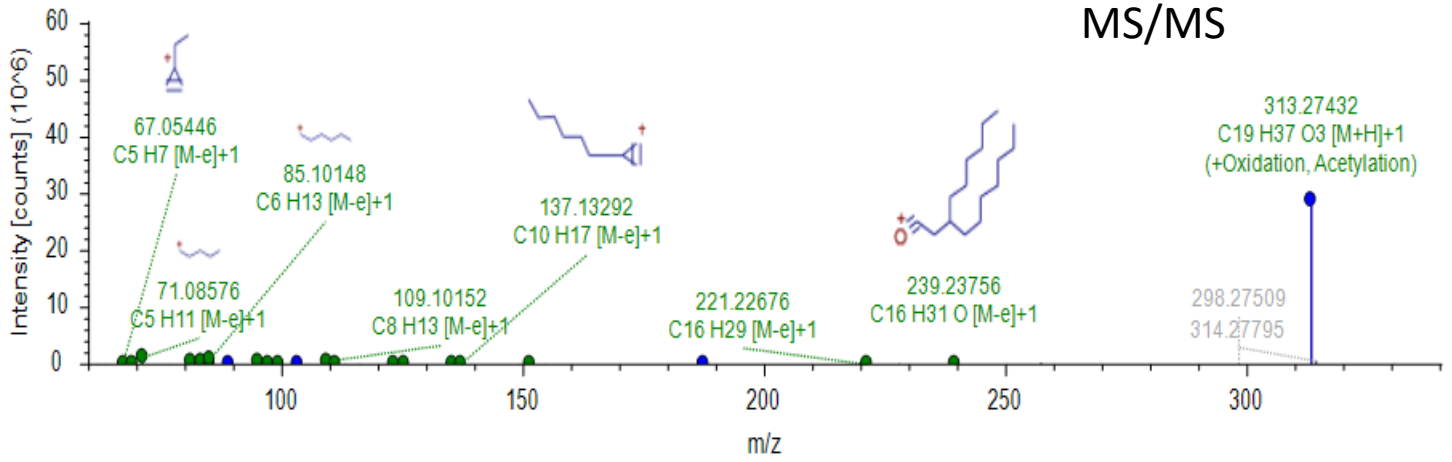


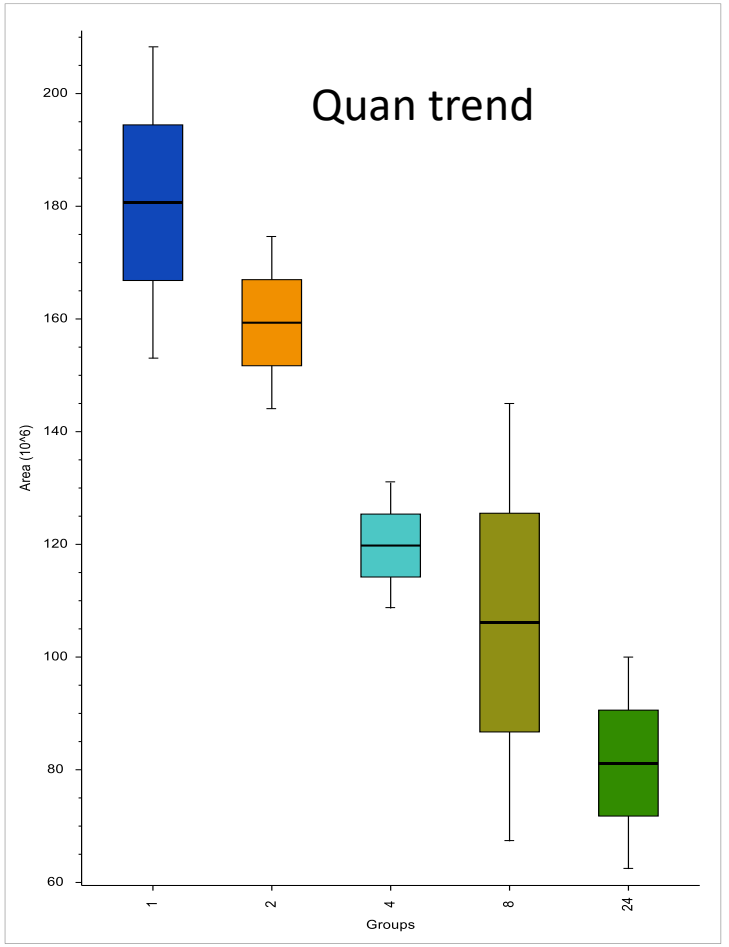


Potential metabolite of SM-102 from the liver samples

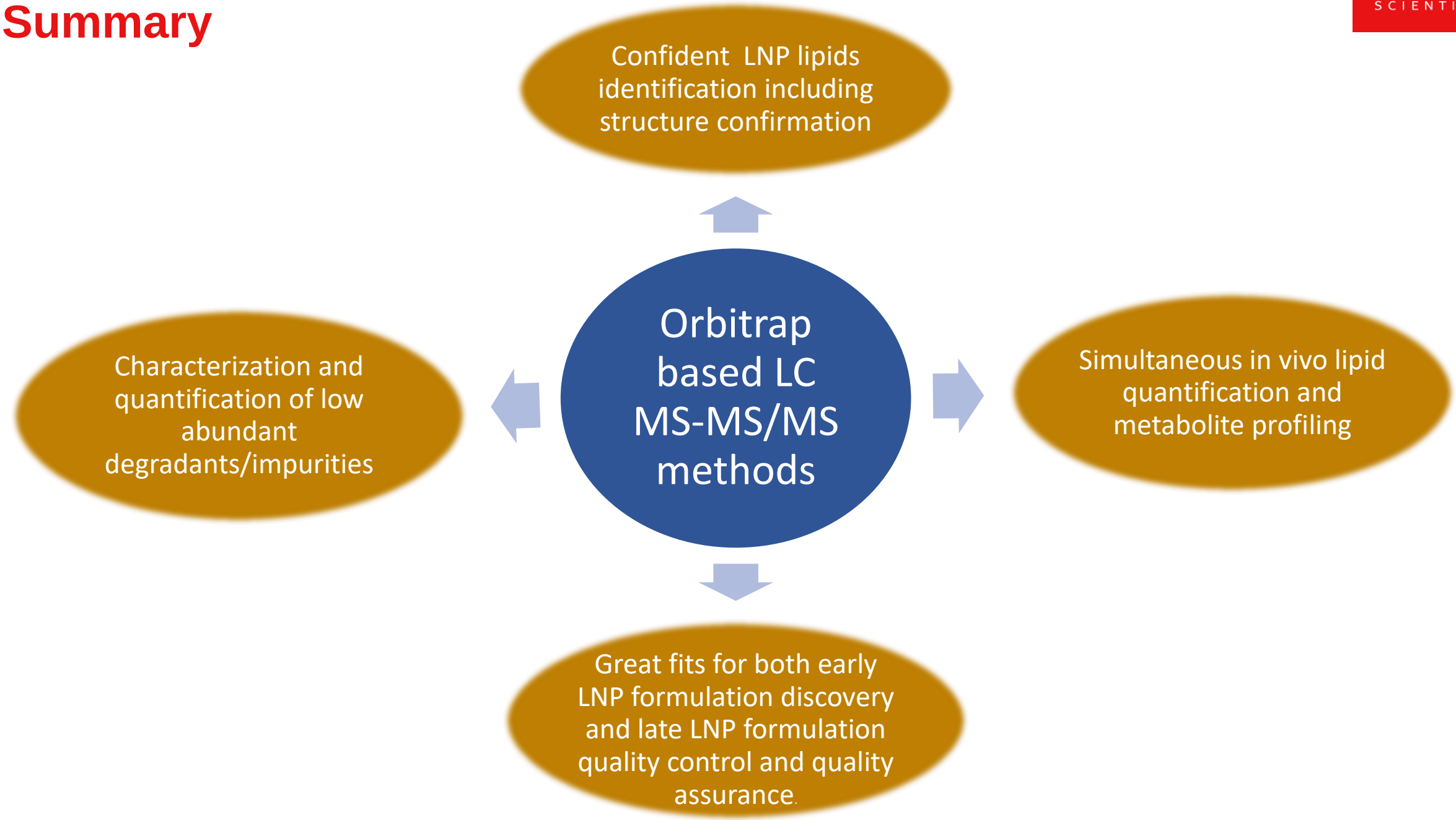
Formula	Parent Compound	Dealkylated	Transformations	Composition Change	Annot. ΔMass [ppm]	Calc. MW	m/z	RT [min]	FISh Covera	Area (Max.)	MS2	Reference Ion
C19 H36 O3	SM-102	X	Oxidation, Acetylation	-(C25 H51 N O2)	-0.65	312.26624	313.27352	2.598	76.67	208193016		[M+H]+1







Summary


Confident LNP lipids identification including structure confirmation
Characterization and quantification of low abundant degradants/impurities
Orbitrap based LC MS-MS/MS methods
Simultaneous in vivo lipid quantification and metabolite profiling
Great fits for both early LNP formulation discovery and late LNP formulation quality control and quality assurance.

Acknowledgment

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- Min Du

Cell Biology Division

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