Identification of reactive N-oxide impurities in LNPs by LC-MS/MS

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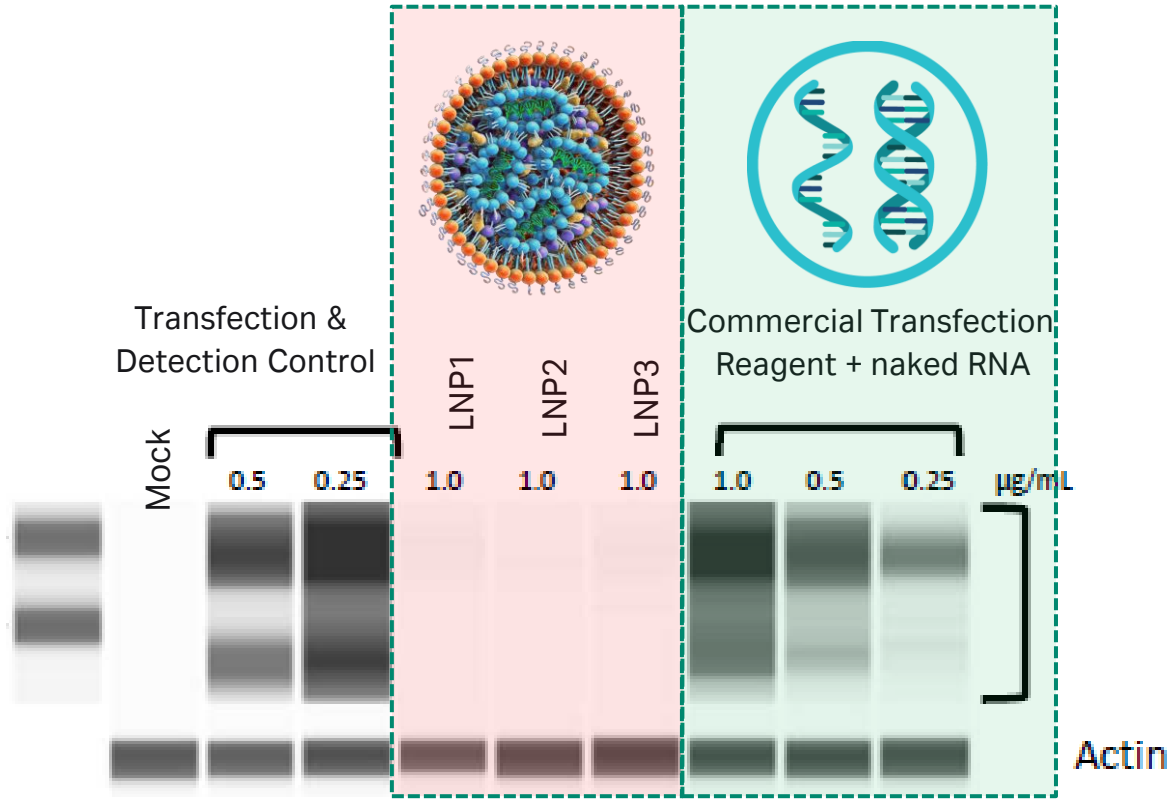
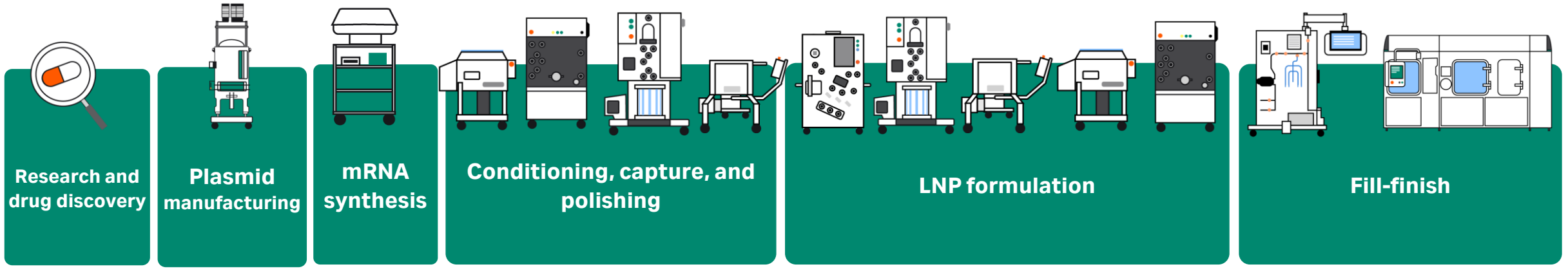
Pre-clinical LNP manufacturing – CASE STUDY

- Preclinical client targeting scale-up for GLP/TOX batch.
- Robust LNP in development.
- Sudden loss of in vitro potency observed in scale-up LNPs.
- Normal potency of neat mRNA payload.
- No obvious change in particle characteristics/ RNA integrity/etc.
- New lipid lot used.



WHAT WOULD LEAD TO TOTAL LOSS OF POTENCY?

Cytiva offers complete solutions for mRNA LNPs



Services and support

From flexible unit operations - to integrated boxes – to manufacturing capabilities

Full stack of technology to enable the genomic medicine revolution

Disease target

A convenient platform to mix and match multiple antigen binding domains.

Genomic medicine toolkit

Genomic payload platform

Synthetic gene delivery not requiring the packaging of a cell line.

Easily adapted to introduce mRNA into a variety of T cell subsets

GenVoy™ delivery platform

Highly durable gene modulation.

Transient nature of the Cas9 mRNA minimizes the risk of off-target edits.

NanoAssemblr™ manufacturing platform

Rapid production of RNA-LNP on demand.

Same proprietary NxGen™ mixing architecture to scale from microliters to hundreds of liters.

Drug development experience

Demonstrated end-to-end drug development capabilities.

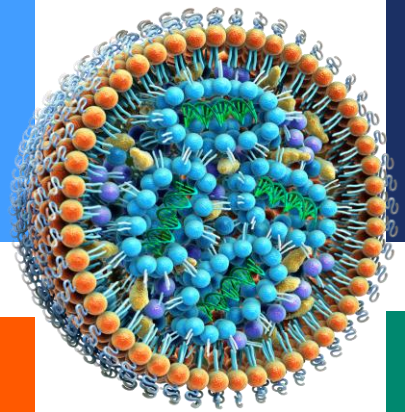
mRNA LNPs: An analytically complex drug product

LNP composition

- Size, PDI, ζ-potential
- Stability
- Lipid composition
- Excipients

RNA quality

- Quantity
- Integrity
- Structure



***In Vitro* Potency Assay**

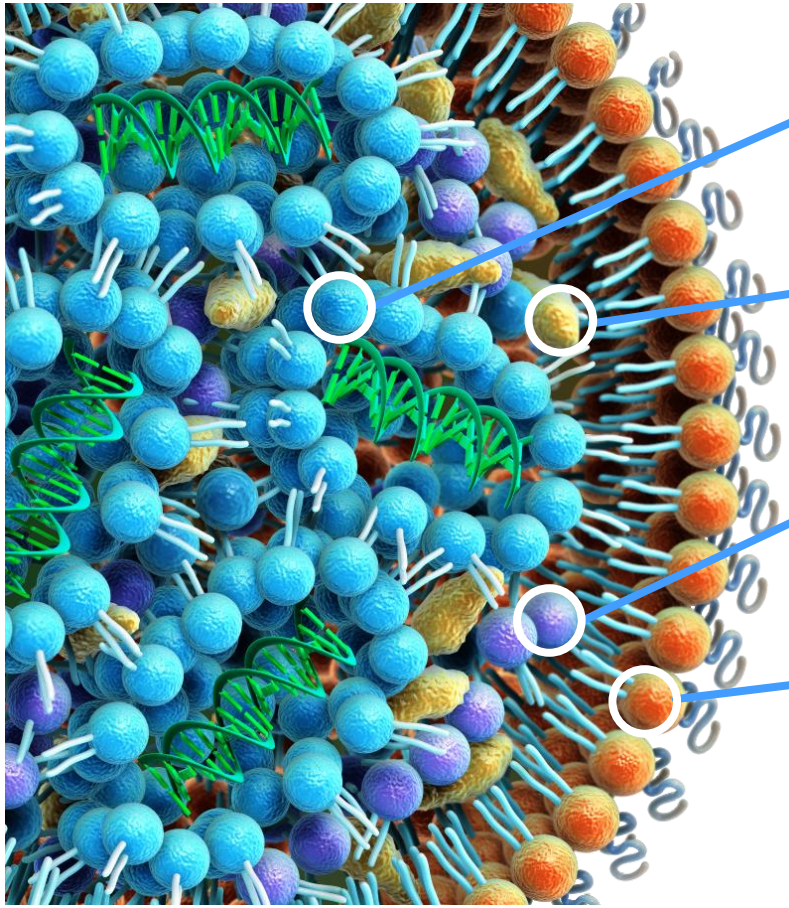
Payload design

- Sequence
- Production
- Biology

Encapsulation

- %EE
- Location of RNA

LNPs contains an analytically complex mixture of lipids



Ionizable cationic lipid

- Neutral at pH 7
- Cationic at pH ~4.5

Cholesterol

- Binds ApoE
- Mediates endocytosis via LDL receptor

Helper lipid (e.g., DSPC, DOPC)

- Fills voids
- Been found to affect release

PEG-lipid

- Stabilizes particle during formation
- Protect from opsonization
- Believed to shed from particle over time following systemic injection

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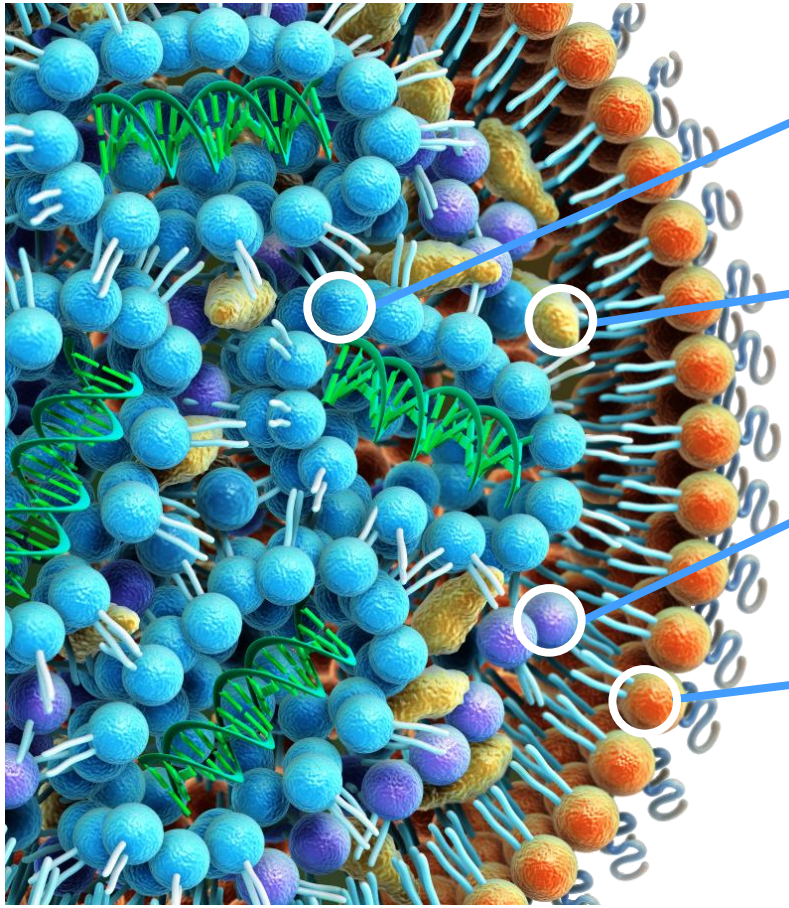

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Analytical challenges

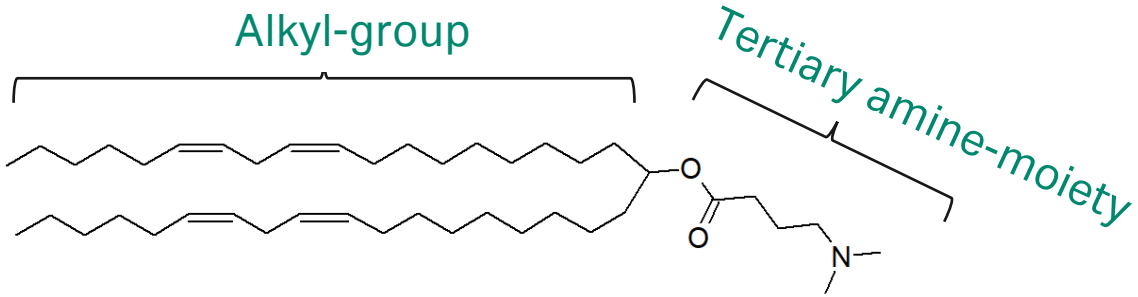
- Numerous....

- Poor ionization in electrospray ionization (ESI)
- Source? Purity? Solubility?

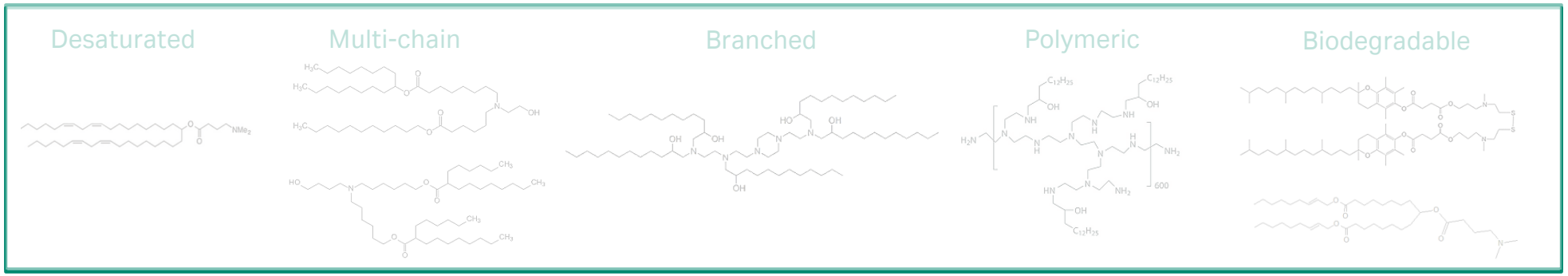
- Stability? Solubility?
- Anionic, liquid chromatograph (LC) peak shape

- Polydispersity
- Complexity/size
- Impurities

Ionizable lipids enable LNP technologies



- Although most contain a tertiary amine, structures range widely for ionizable lipids.
- Recent trends include biodegradable lipids, complex alkyl groups, and polymeric species.



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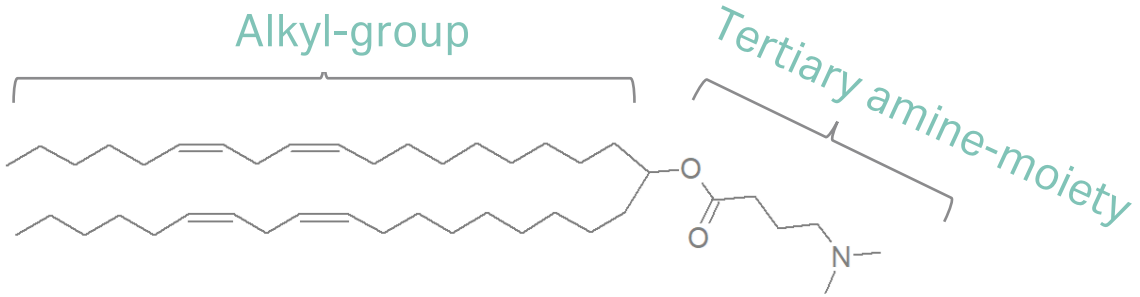

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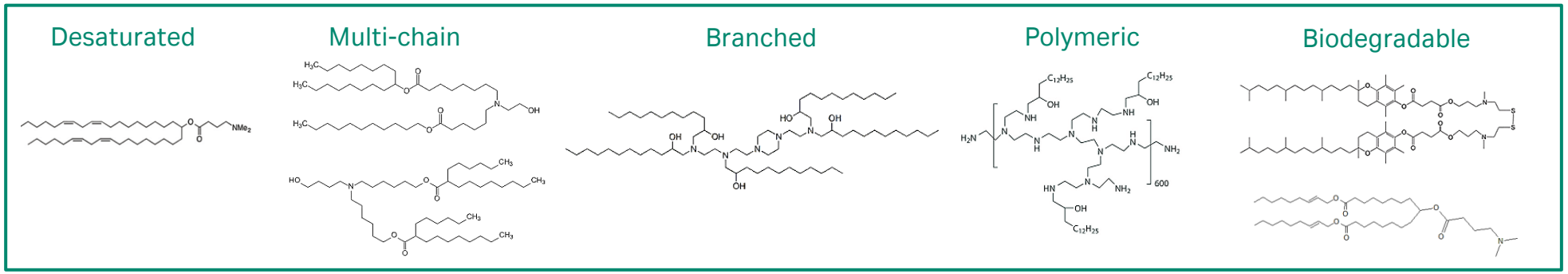
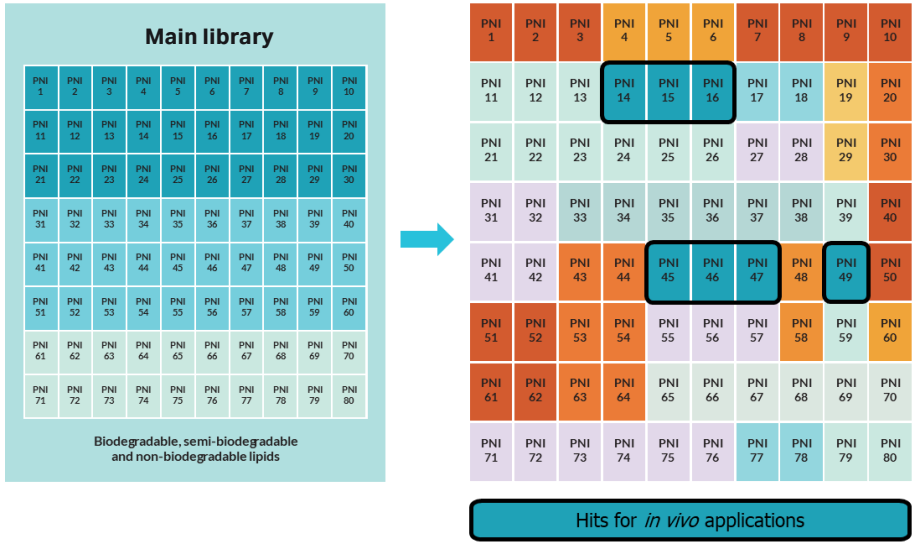


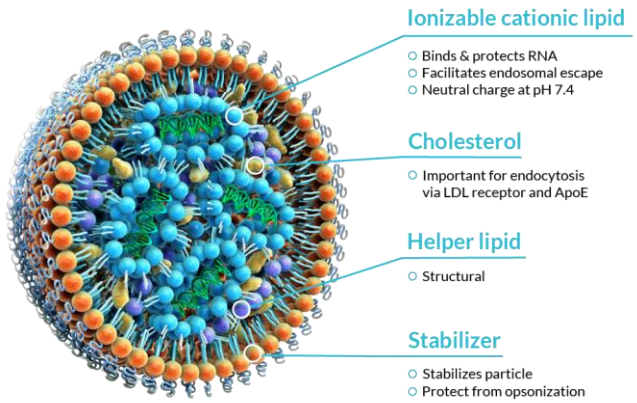
GenVoy™ platform: lipid nanoparticle delivery

- Proprietary LNP technology and associated intellectual property
- Novel lipids and compositions designed and optimization for established use cases (i.e., vaccine, gene therapy, cell therapy) as well as emerging applications

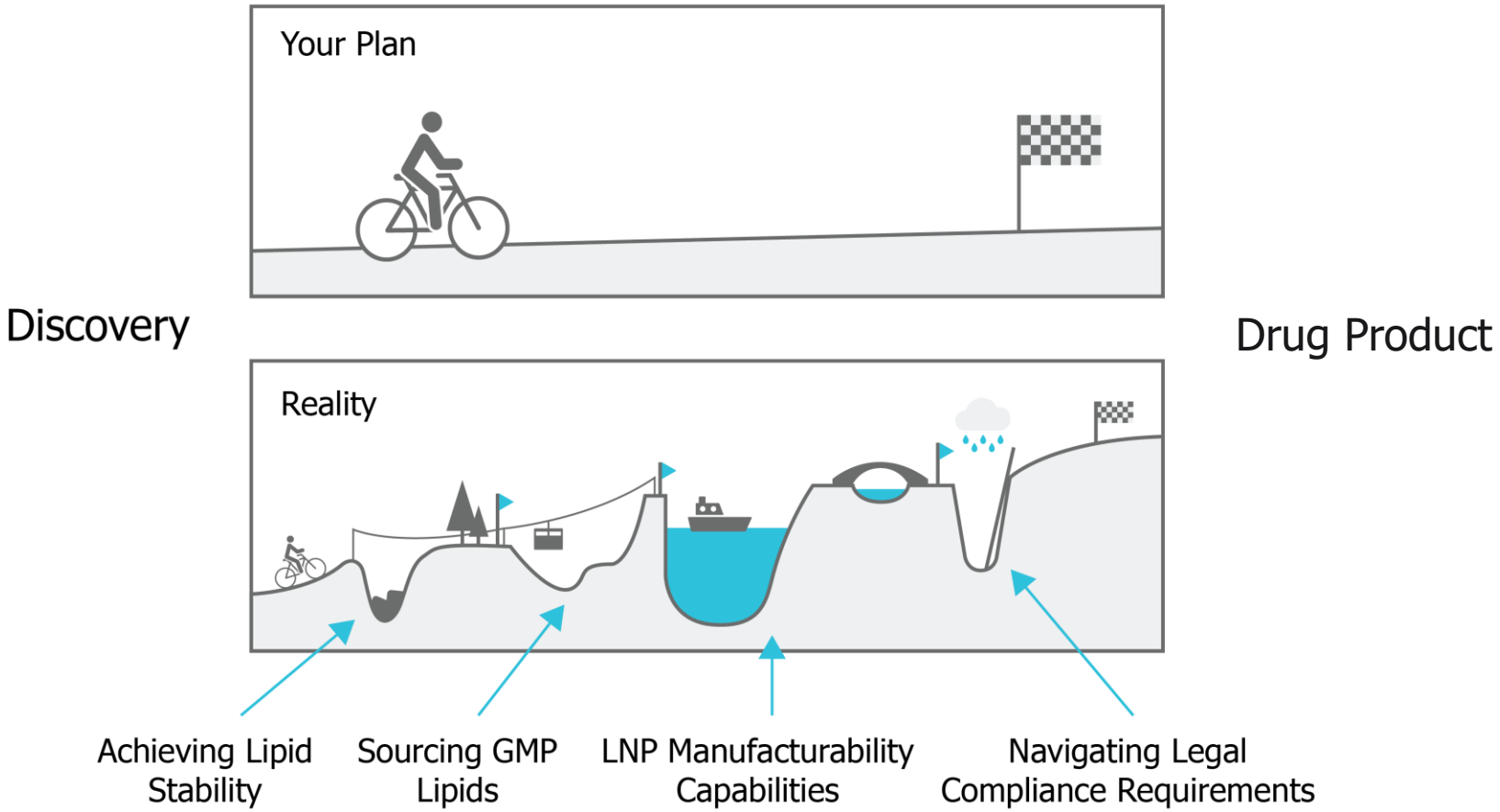
Diverse library of novel ionizable lipids



Novel compositions designed for specific applications



Navigating the realities: From Discovery to Scale-up



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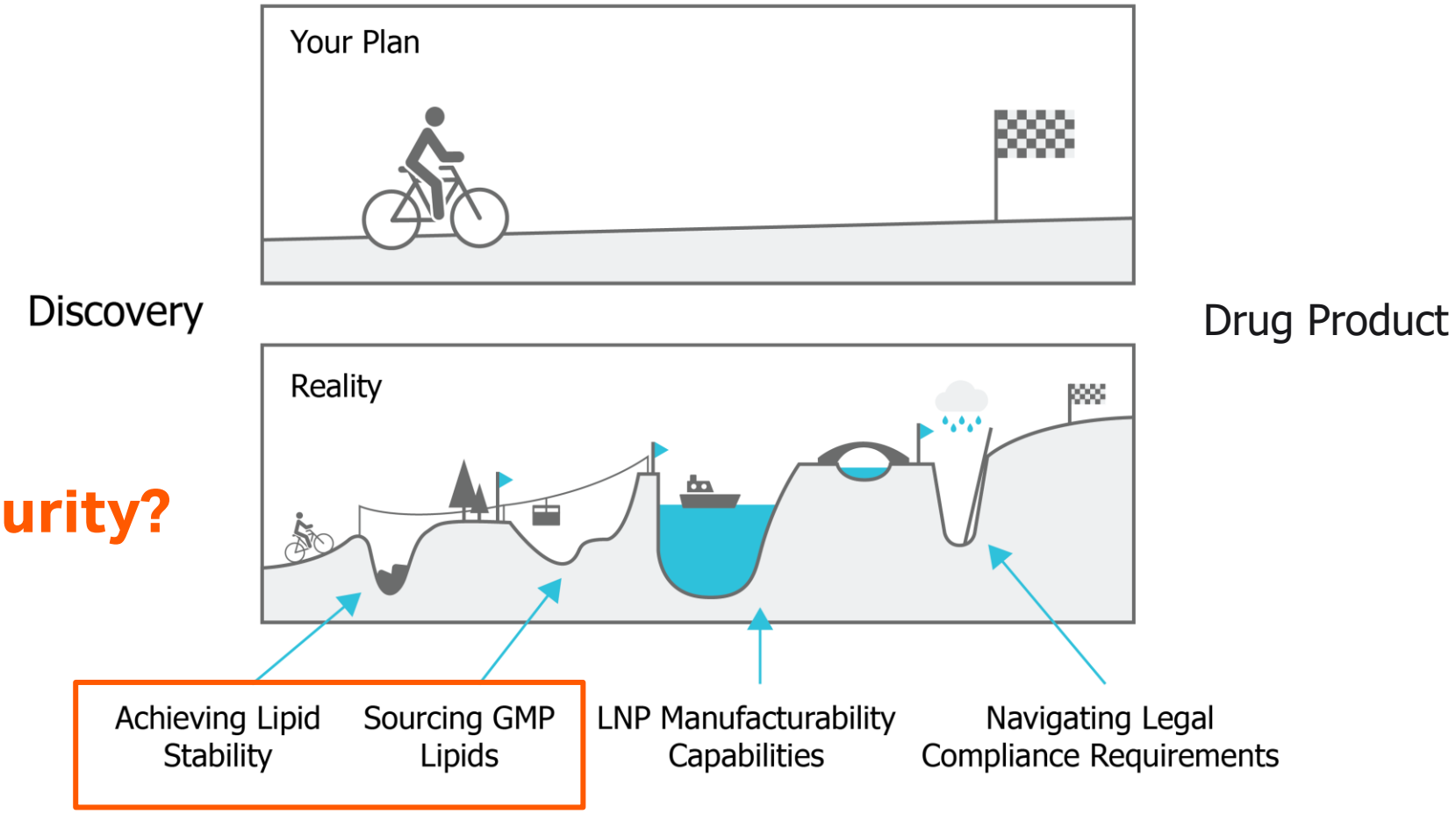
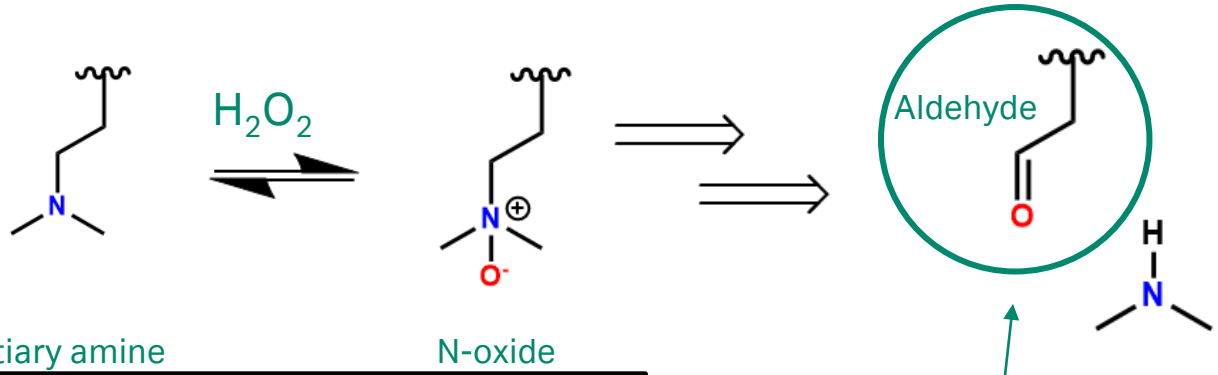
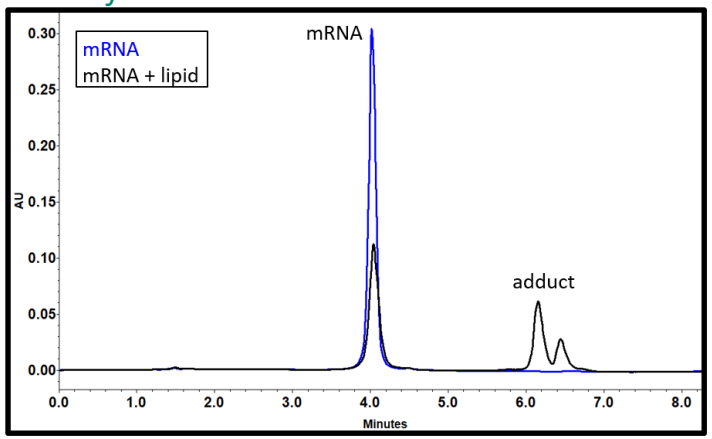


Lipid impurities present risks for LNP formulations





Reactive with amines or hydroxyls in ribonucleosides

- Tertiary amines in iLs oxidize to N-oxide impurities which are thought to degrade into reactive aldehydes(1).
- Aldehydes crosslink with nucleoside bases forming LIPID ADDUCTS.
- Cytiva has developed assays to directly monitor mRNA lipid adducts.

1. Packer M, Gyawali D, Yerabolu R, Schariter J, White P. A novel mechanism for the loss of mrna activity in lipid nanoparticle delivery systems. Nature News. November 22, 2021. Accessed March 30, 2024. https://www.nature.com/articles/s41467-021-26926-0

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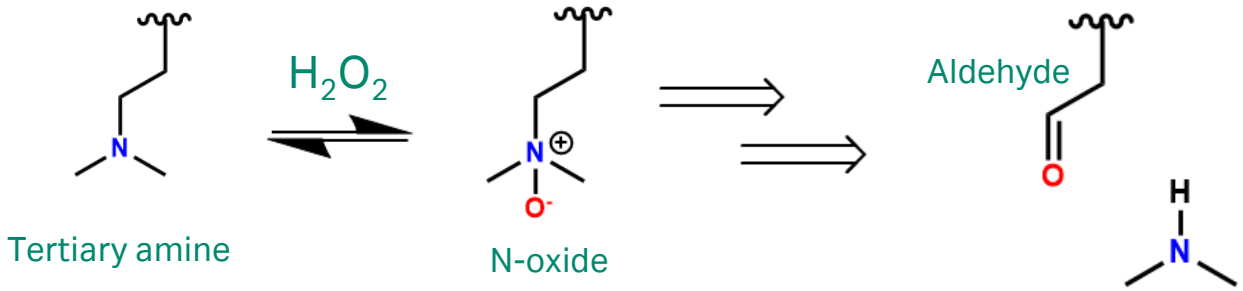

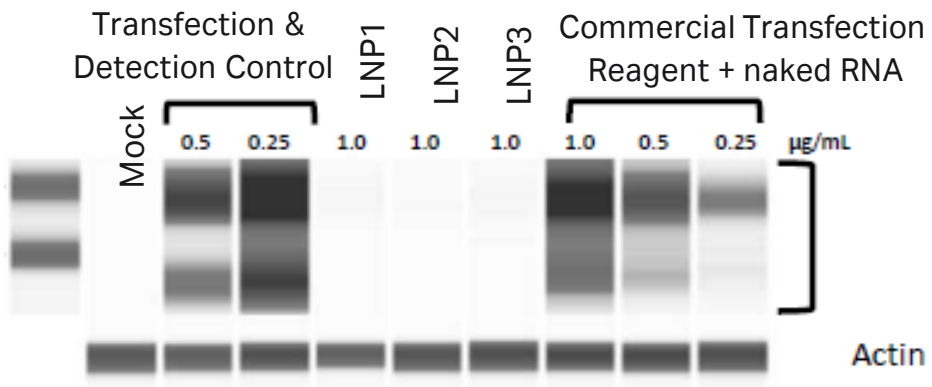

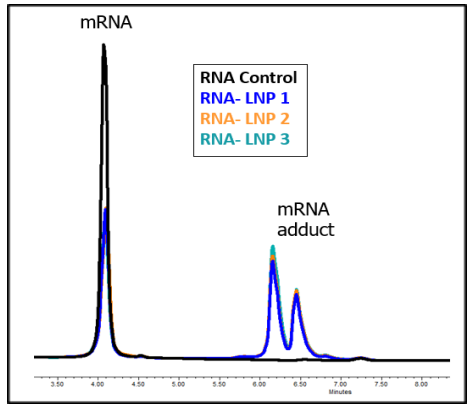

Lipid adducts appear to be the source of inactivation in these LNPs.

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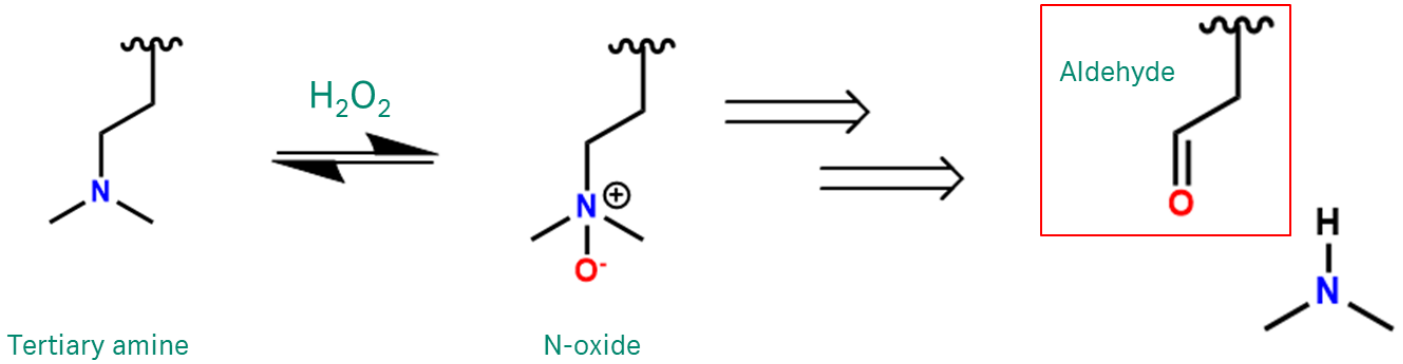

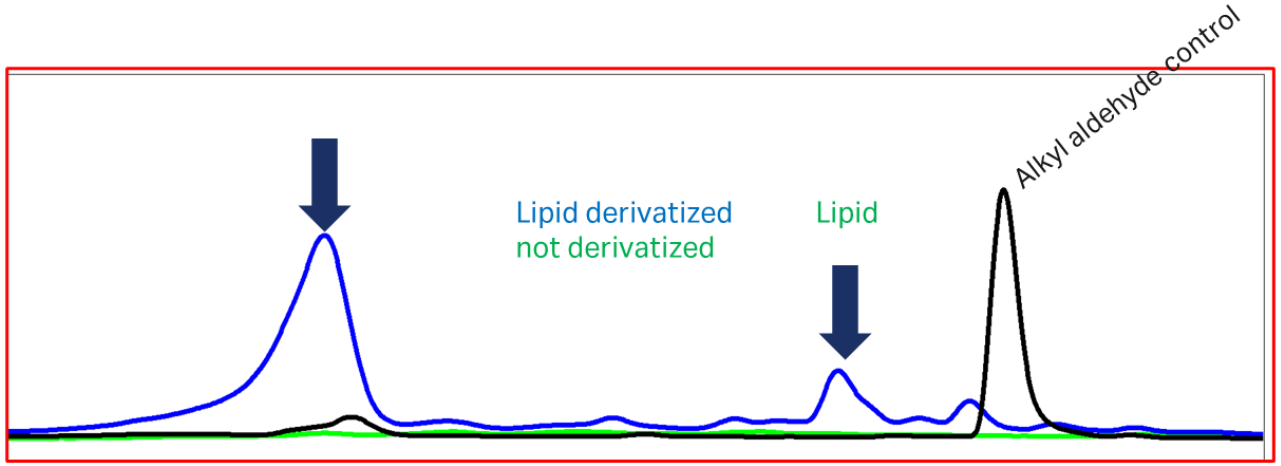

- Aldehydes detected in ionizable lipid stocks.
- Hydrophobicity consistent with degradation of N-oxide from iLs.

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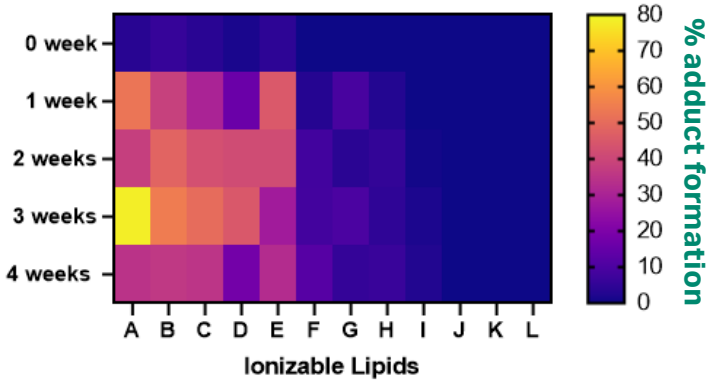
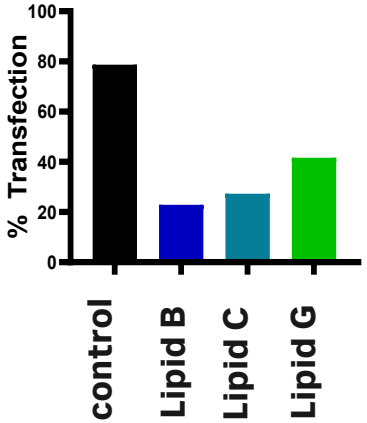
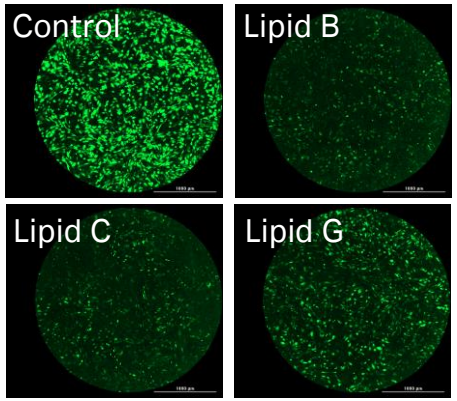
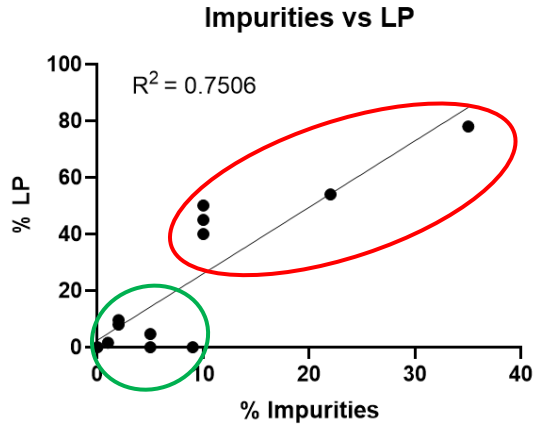
Q: How do we predict if adduct formation is likely?





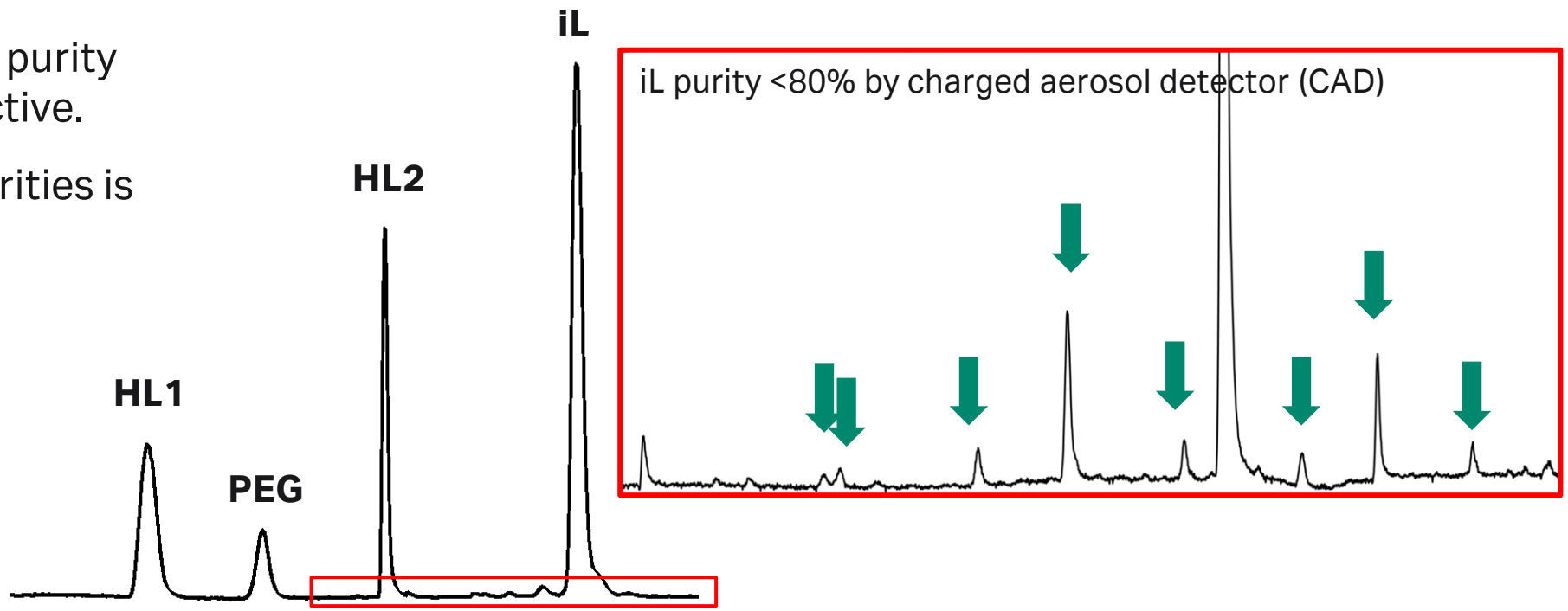




- 12+ unique iLs screened for adduct formation.
- Monitored aldehyde formation, integrity, *in vitro* potency, etc.
- No simple correlation was observed across iL structure (pKa, size, etc.), aldehyde content, RNA stability, etc.
- HOWEVER, the largest prediction of adduct formation was LIPID PURITY.

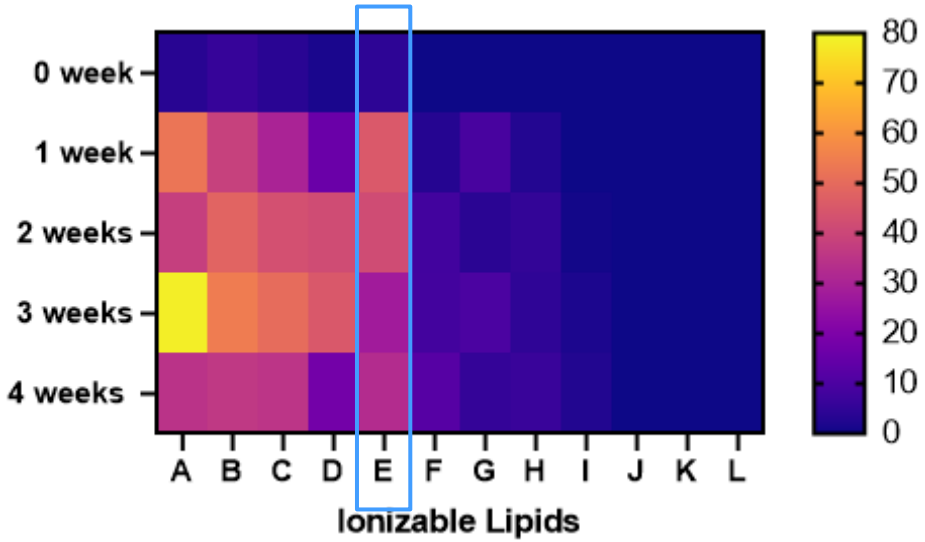
Not all impurities are equal

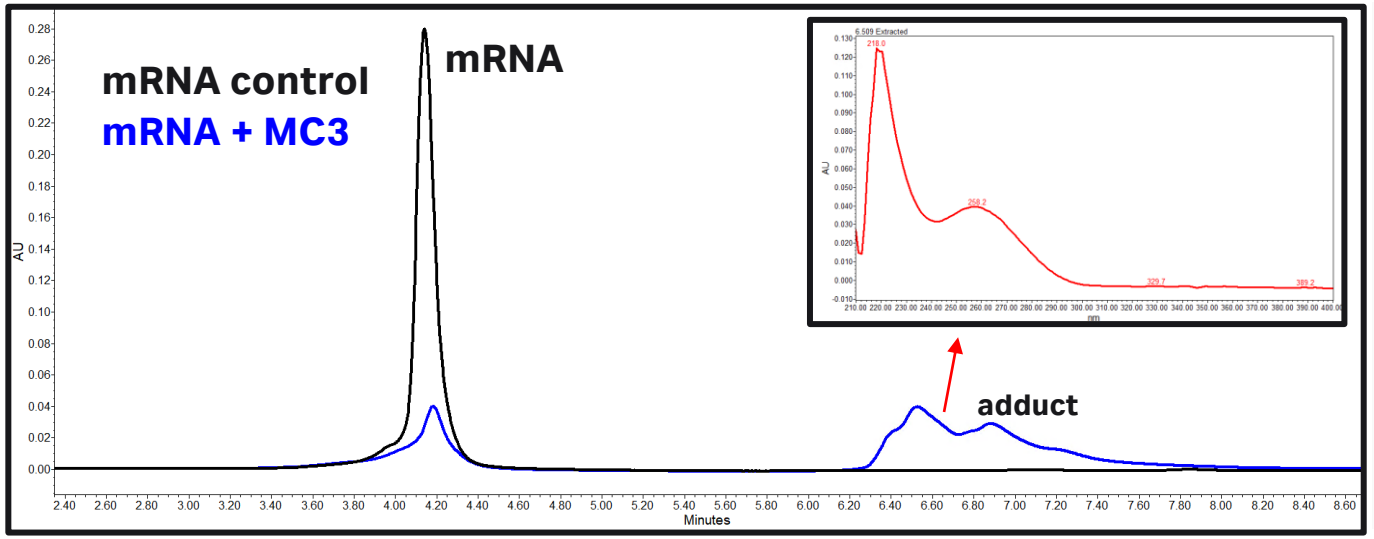
- Adduct formation is correlated with lipid purity but not solely predictive.
- Assignment of impurities is paramount.



Profiling lipid impurities by high-resolution mass spectrometry (HRMS)

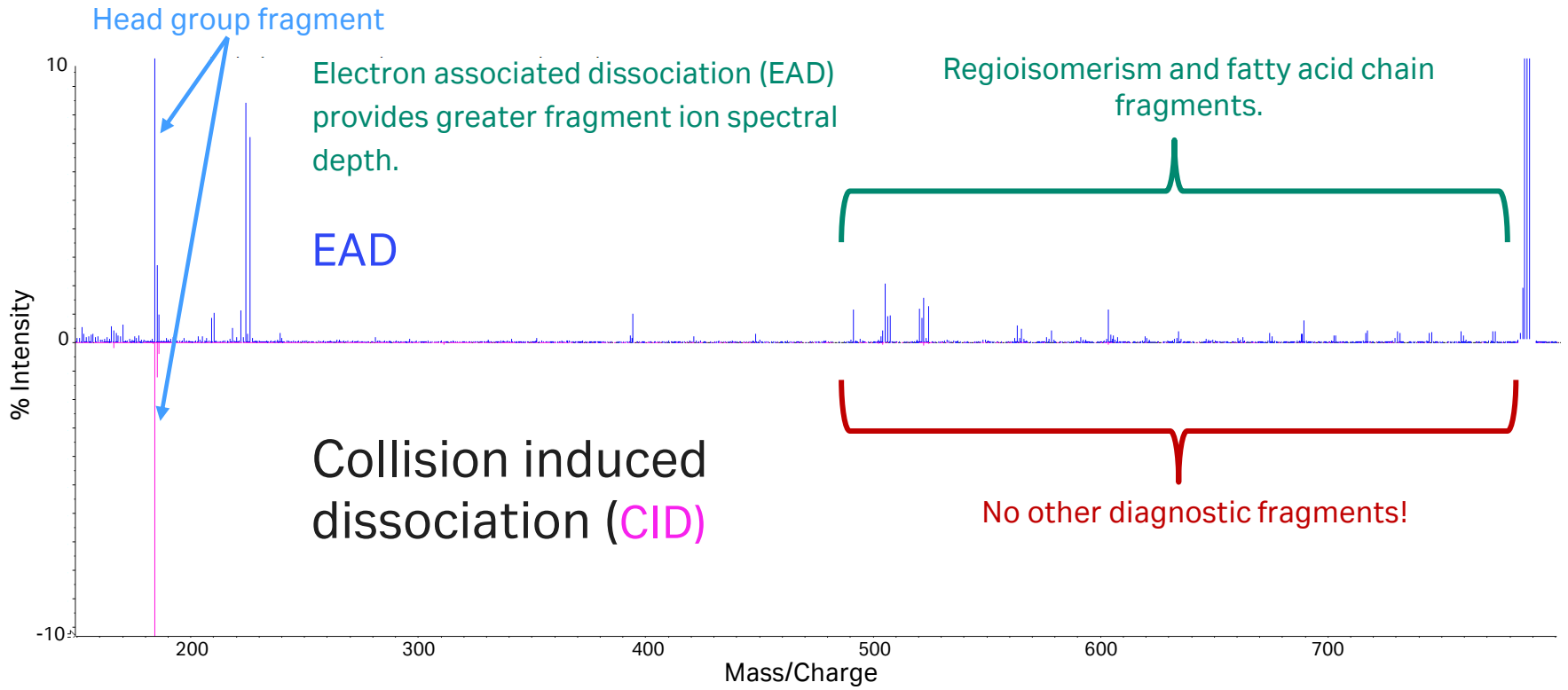
- Model ionizable lipid DLin-MC3-DMA (MC3) included in screen as LIPID E.
- Purity by UHPLC-CAD ~ 90%.
- At its peak, MC3 resulted in 30-40% mRNA adduct (in binary system, not LNP).





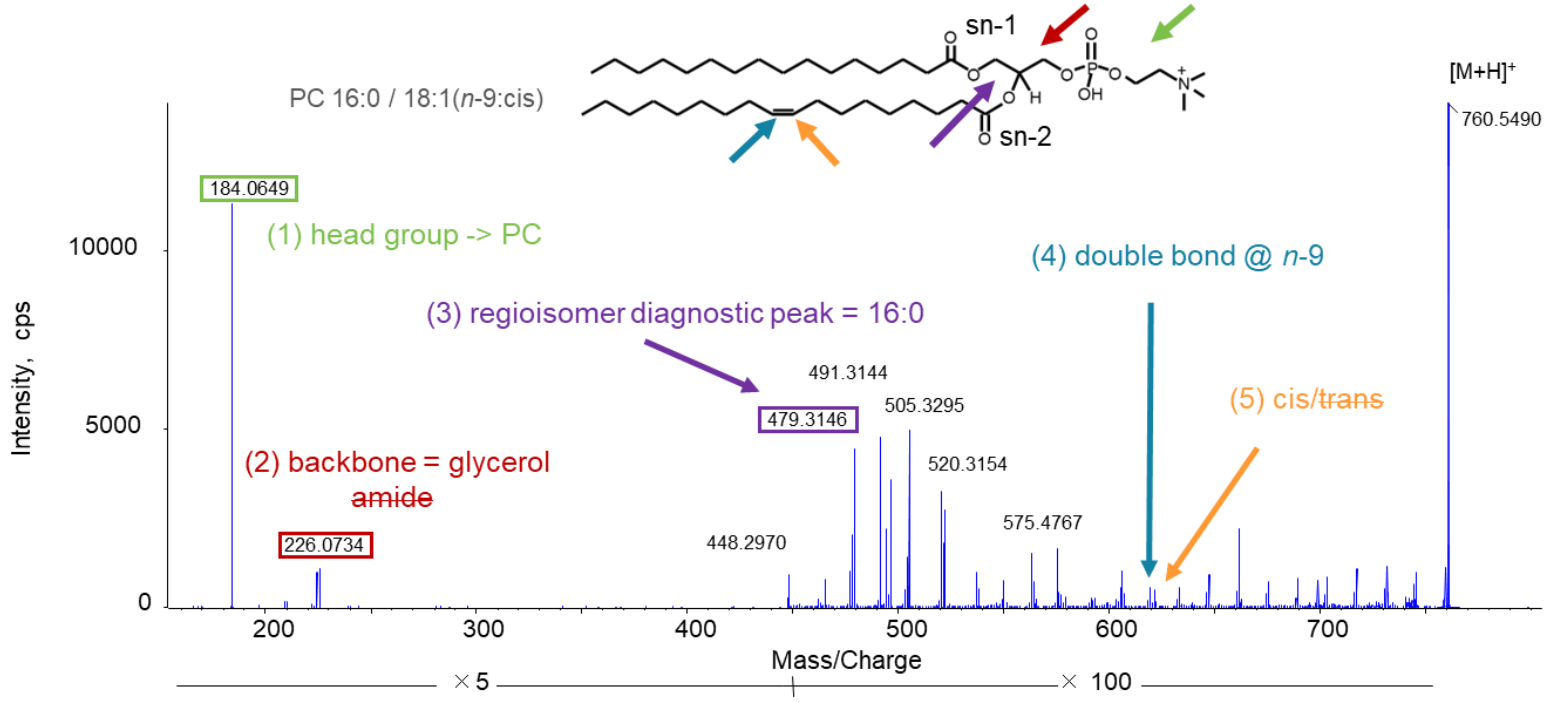
Digging deeper into the molecule

MS/MS spectra of Phosphatidylcholine (PC) with different fragmentation modes.



Bringing lipid characterization to the next level

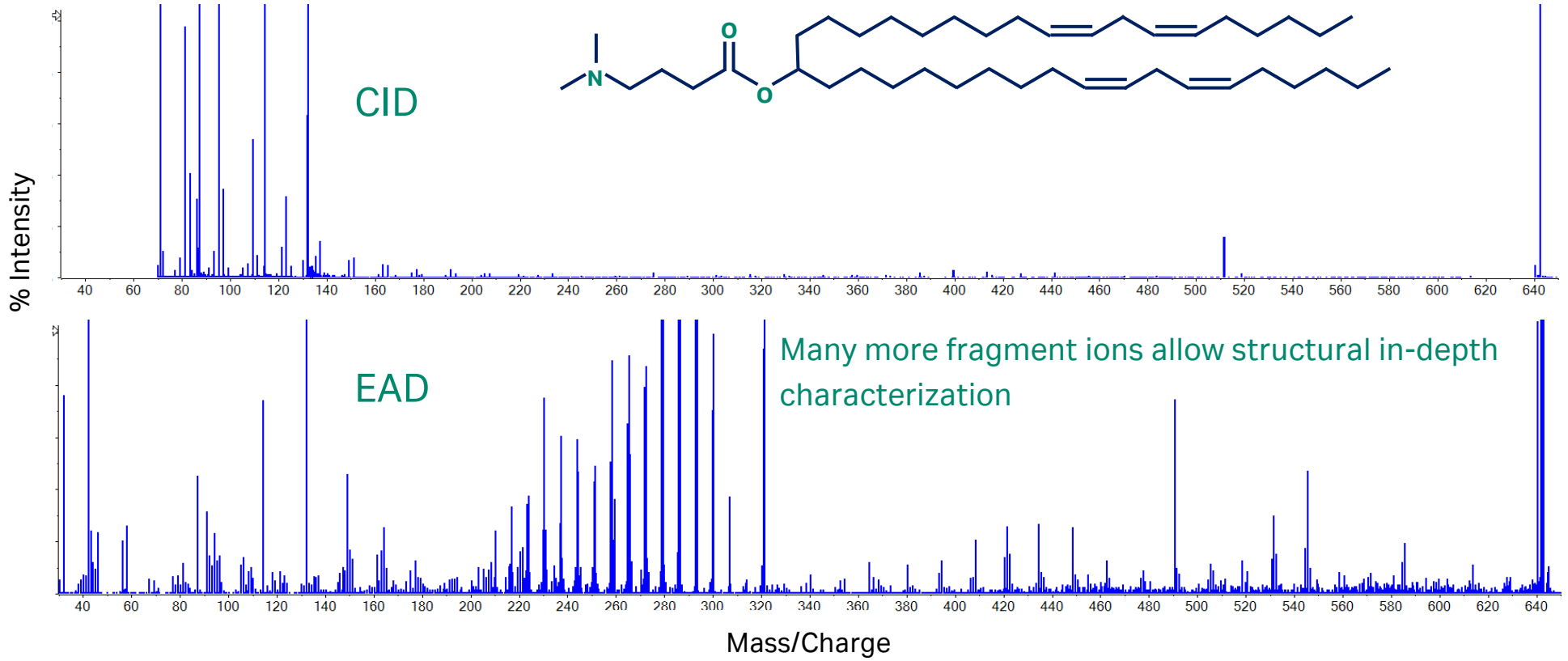
- The exact structure of a phospholipid can be determined within a single LC-MS/MS run by using EAD



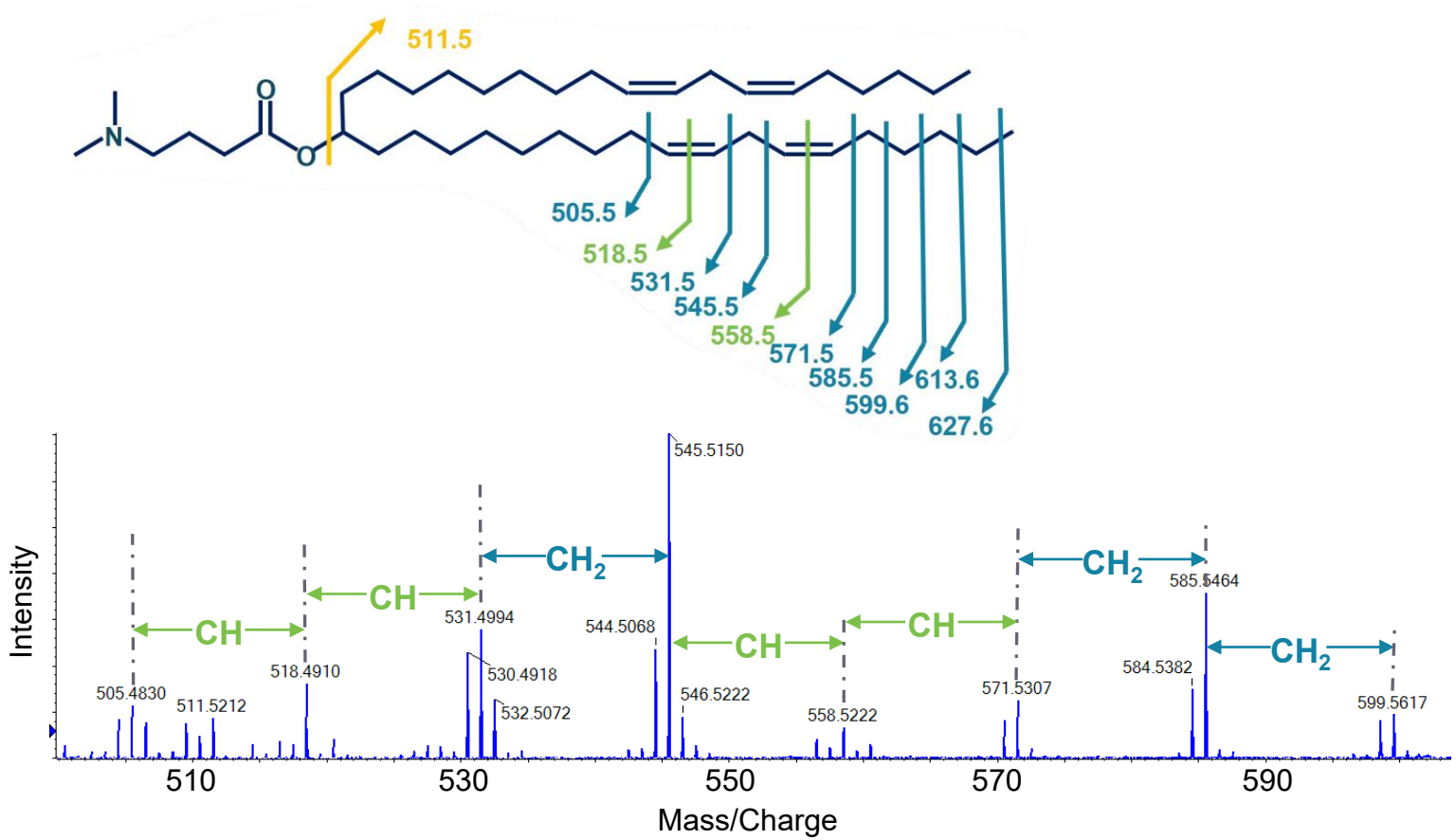
2. J. Larry Campbell and Takashi Baba *Analytical Chemistry* **2015** *87* (11), 5837-5845DOI: 10.1021/acs.analchem.5b01460

Applying EAD to ionizable lipids used in LNPs

Zoom-in of MS/MS spectra of DLin-MC3-DMA (MC3)



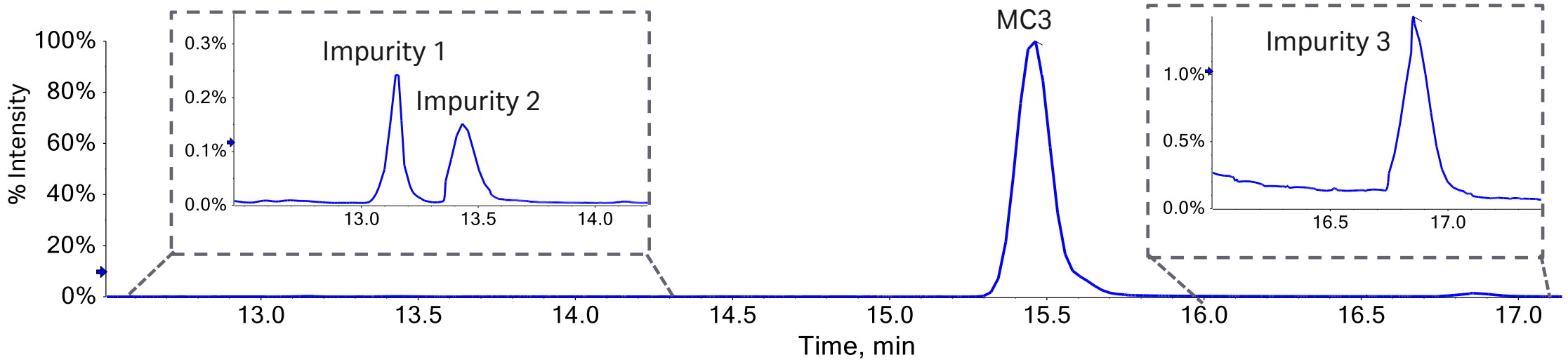
Alkyl chain characterization of MC3



- Every carbon-carbon bond is cleaved by EAD.
- Double bonds within MC3 can be determined.

MC3 and related impurities

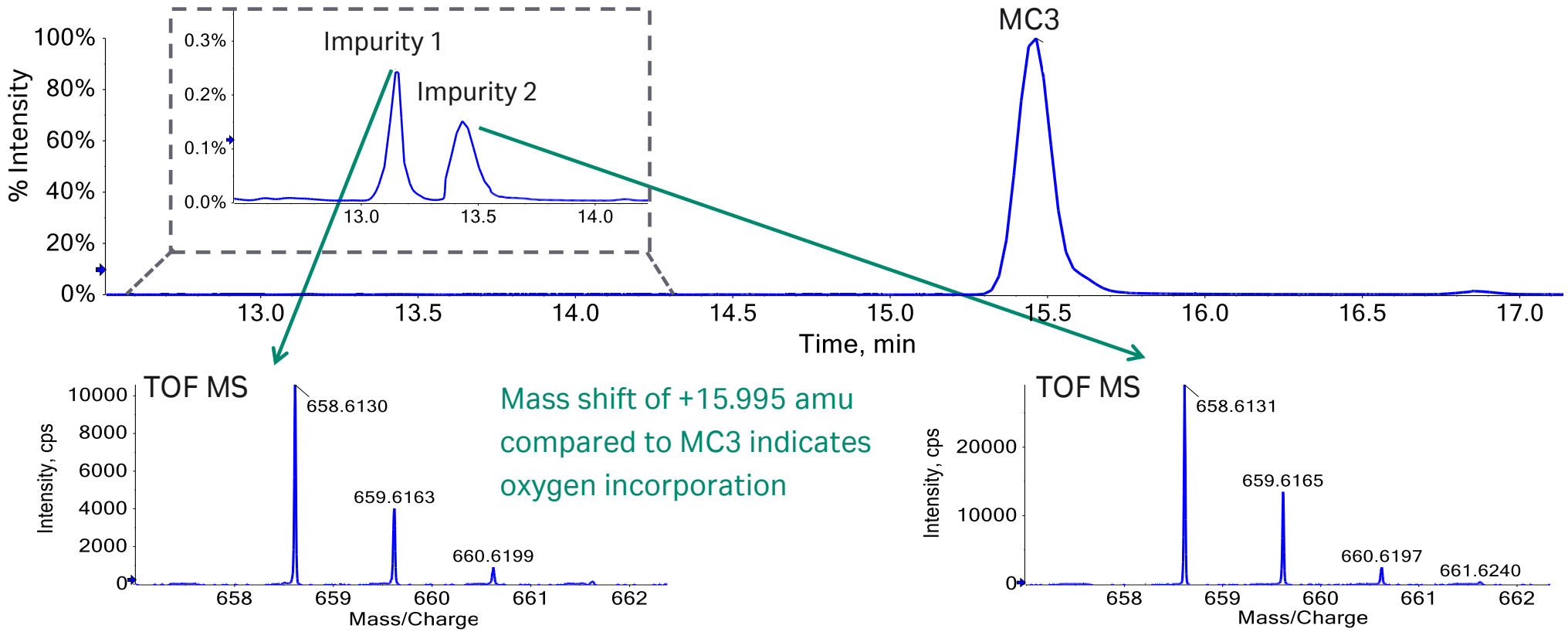
Extracted ion chromatograms (XIC)



Several low-abundance impurities were detected in the MC3 preparation.

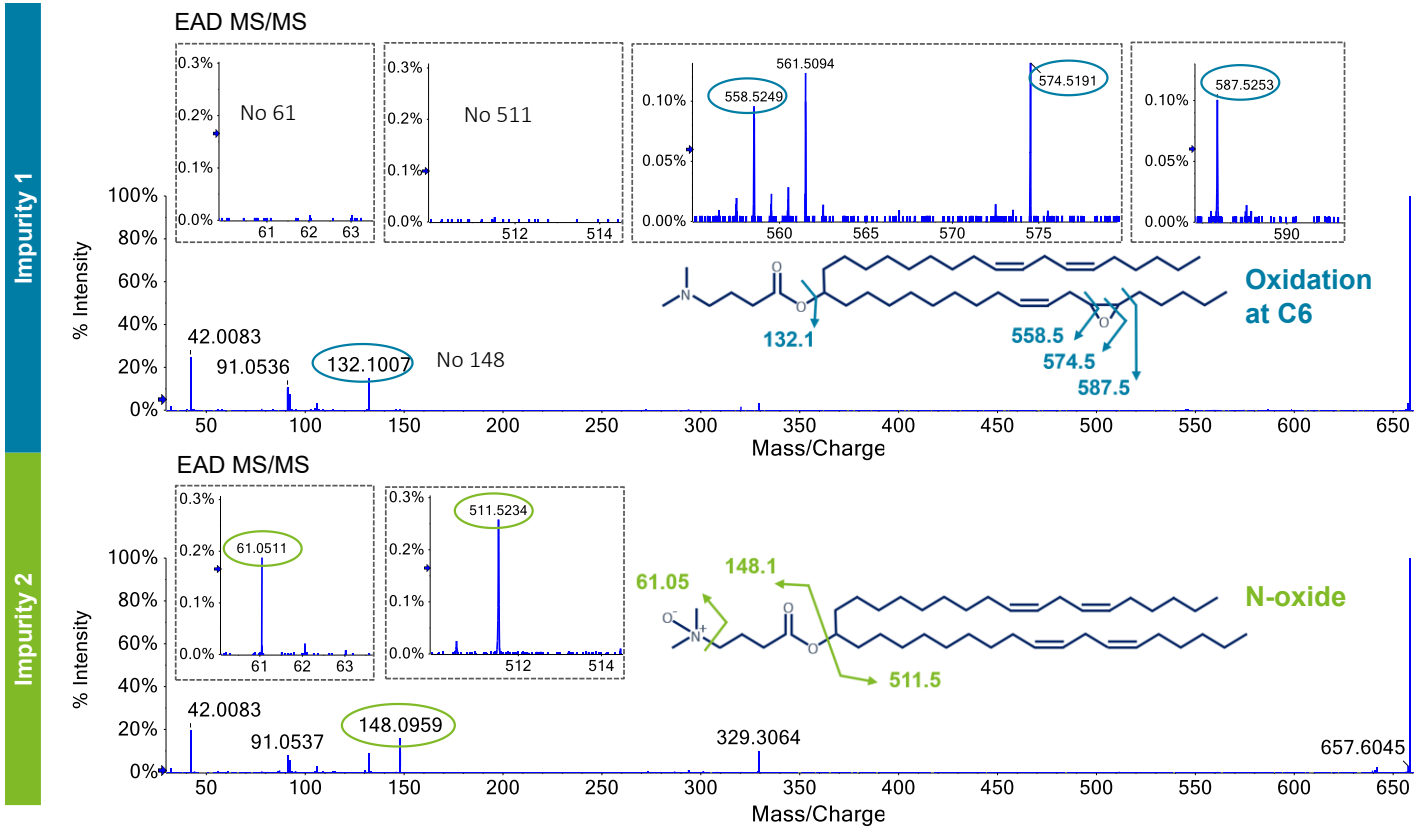
Impurity 1 and 2: Oxygen incorporation

But where in the molecule is the Oxygen?



Localization of oxygen incorporation

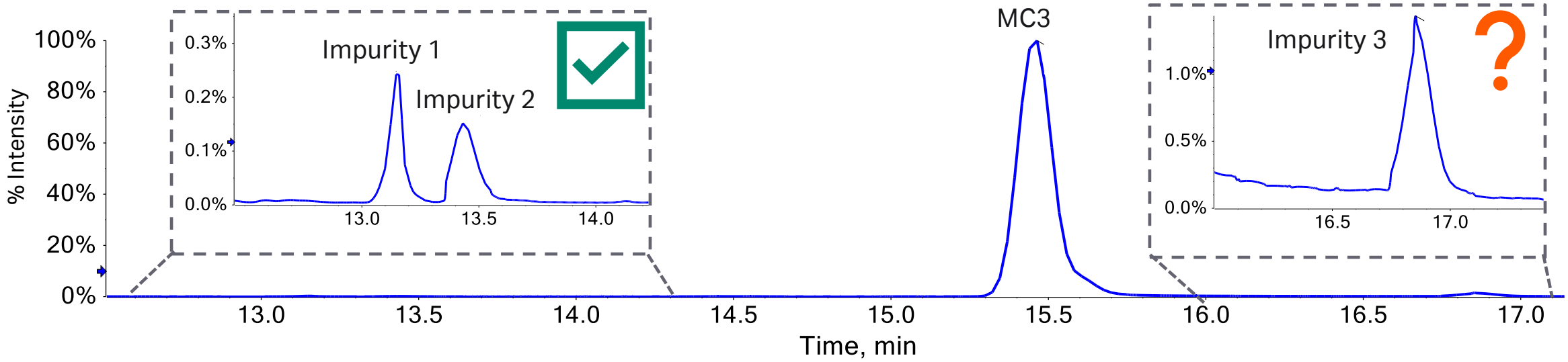
With EAD



- EAD provides a higher amount of descriptive fragments.
- Impurity 1: unique fragment ions at *m/z* 558.5 and 574.5 pinpoint oxygen incorporation to C6.
- Impurity 2: unique fragment ion at *m/z* 61 identified impurity as the N-oxide.

MC3 and related impurities

Extracted ion chromatograms



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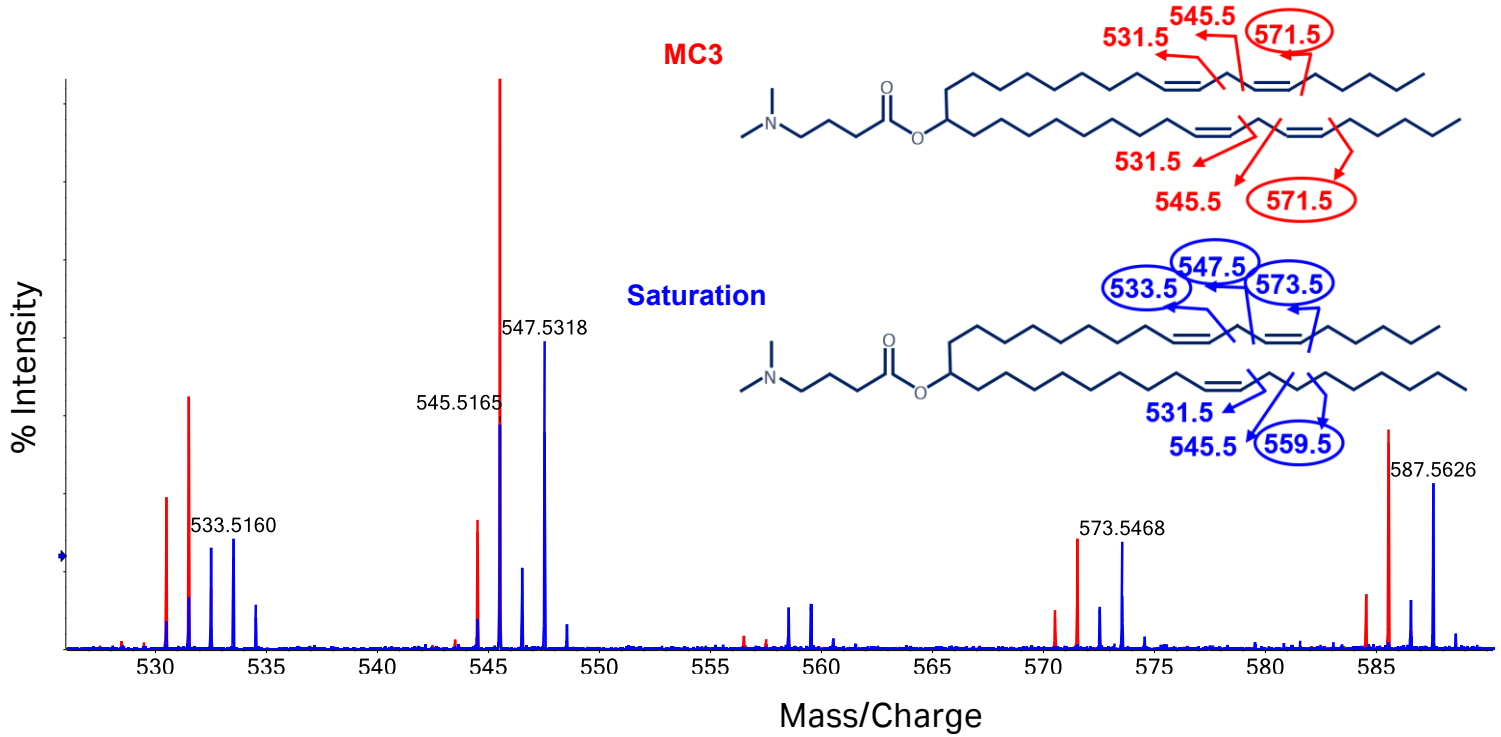
Impurity 3: Localization of saturation

EAD MS/MS spectra



- Impurity 3 showed a mass shift of 2 amu compared to MC3.
- Exact localization of the saturation of one of the double bonds at position C6 based on detailed fragmentation of the alkyl chains with EAD.

MC3 impurities characterized by Zeno EAD

Compound	Relative abundance [%]	*m/z* error [ppm]
MC3	*97.9*	*0.2*
Saturation at C6 of alkyl chain	*1.11*	*-1.1*
Desaturation at C3 of alkyl chain	*0.41*	*-0.5*
N-de-methylation	*0.19*	*-1.6*
Oxidation of head group	*0.15*	*-0.3*
De-methylation of alkyl chain	*0.10*	*-1.3*
Oxidation at C6 of alkyl chain	*0.06*	*-0.4*
De-ethylation of alkyl chain	*0.03*	*-3.2*

- Impurities well below 0.1% were identified and characterized within a single injection.
- Exact positions of modifications could be determined with Zeno EAD.
- Relative quantification was performed simultaneously using MS1 XIC peak areas.

Summary

- RNA-lipid adducts appear ubiquitously across LNP compositions however their formation does not correlate well with the chemical properties of different ionizable lipids
- Initial data suggests ionizable lipid purity being the largest predictor of adduct formation
- Lipid impurities can affect LNP formulation integrity and mRNA activity, even if present at very low abundances.
- Identification of impurity species is key to ensuring high product quality, but existing technologies struggled with detailed structural elucidation.
  - Many more fragments can be obtained with EAD compared to CID, allowing exact localization of oxidation and double bonds in ionizable lipids.
  - Differentiation of concerning N-oxides from other oxidation-related impurities can be achieved with confidence at very low abundances.

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