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AQbD-Based Method Development for the Analysis of Lipid-Based Nucleic Acid Delivery Systems Using ChromSwordAuto Software

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Abstract

A UHPLC method was developed for the analysis of lipid-based nucleic acid delivery systems and their building blocks using an automated Analytical Quality by Design (AQbD) approach. ChromSwordAuto software and corresponding smart algorithms were used to facilitate the process. A design space was generated using Design of Experiment (DoE) and chromatographic resolution as the analytical target profile (ATP). The developed method is based on Agilent ZORBAX StableBond CN columns, and shows baseline separation between all peaks, as well as excellent method robustness for different vehicle types.

Introduction

RNA-based products have been established as the default technology for global vaccination. As the scientific field of RNA technology is wide, the production processes and control strategies are heterogenous and complex. A more rigid regulatory guideline for RNA-products has yet to be established for ensuring product quality.[1,2] One way to create a deeper knowledge of both product and manufacturing is to apply Quality by Design (QbD) throughout the complete RNA platform lifecycle.[3] An elemental piece of the process is analytical method development, through which quality principles described in the ICH guidelines can be implemented. The application of a systematic AQbD approach to analyze this new class of carrier systems could be a paradigm shift, and can aid in securing constant product quality.

Stage 1 procedure design

Fundamental to the concept of QbD is that increased testing alone is not sufficient to support product quality. Therefore, risk assessments and DoEs are used to investigate parameters influencing method performance. The output of DoE identifies a region of reliable operating conditions for the method, referred to as the Method Operable Design Region (MODR). Approaches to the Stage 1 procedure design have been described in the ICH guideline and elsewhere.[4,5]

Typically, the procedure design follows the steps shown in Figure 1. After an initial knowledge gathering about chemical structures, physicochemical properties, and any other relevant information, the ATP is defined. The ATPs shown in Figure 1 represent the performance requirements for this method. Critical quality attributes (CQAs) suitable for the method being developed are then defined and used for a first risk assessment. Relevant CQAs for lipid-based RNA vehicles are lipid content and lipid identity. Specific analytical methods, including acceptance criteria, have yet to be defined by regulatory frameworks.[3]

The critical process parameters (CPPs) are the parameters that influence method performance the most. These are varied in a multivariant approach during data generation. Applying DoE with ChromSwordAuto together with the accompanying smart algorithms enables fast data acquisition and the design of a reliable MODR. DoE is just one type of chemometric tool available. Others include predictive modeling, and other mathematical tools that enable pattern recognition.

Stage 1 procedure design results in enhanced knowledge, which offers flexibility with regard to regulations, simplifying both post-approval changes and root cause analysis.

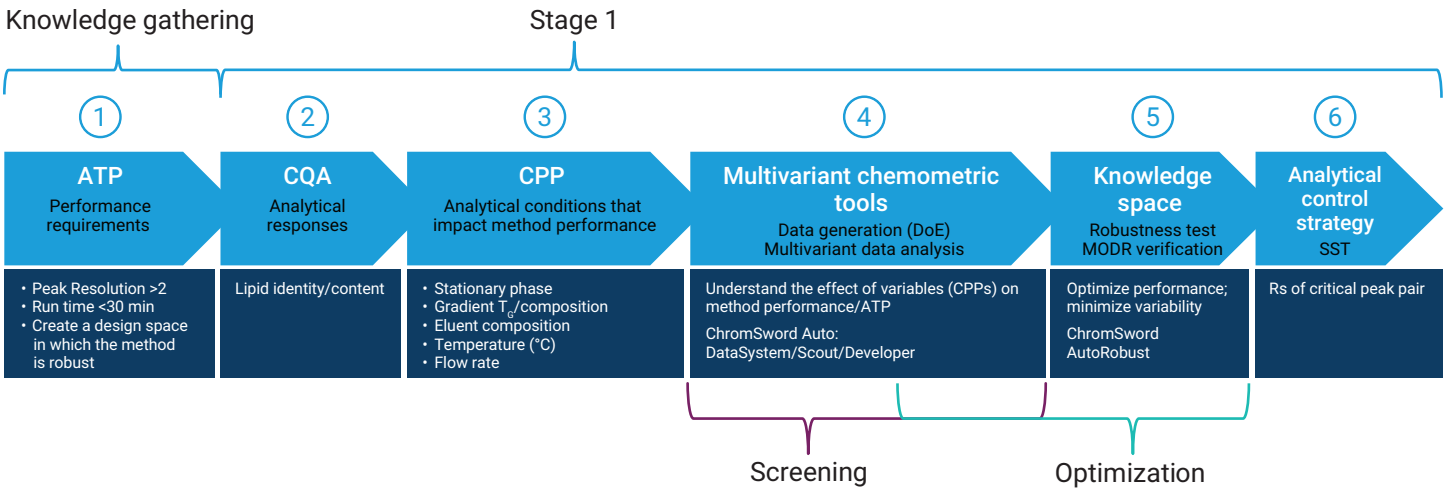


Figure 1. Stage 1 procedure design. ATP: Analytical Target Profile; CQA: Critical Quality Attribute; CPP: Critical Process Parameters; DoE: Design of Experiments; MODR: Method Operable Design Region; SST: System Suitability Test.

Lipid nanoparticle-relevant knowledge gathering

Lipid nanoparticles (LNPs) are typically composed of four main components: cholesterol, a neutral phospholipid (mostly distearoylphosphatidylcholine (DSPC)), an ionizable lipid, and a polyethylene-glycol (PEG) lipid.[6,7] These structural lipids control the particle in terms of size, structure, stability, nucleic acid encapsulation efficiency, cellular uptake, and endosomal escape.[8]

The formulation of the LNP composition and the design of the ionizable lipids have undergone numerous enhancements.[8] This results in various delivery system compositions, with a tendency toward decreasing amounts of DSPC and increasing amounts of newly designed ionizable lipids. In contrast, some of the older delivery formulations contained up to 85 mol% phospholipid.[9] Formulations such as liposomes and stabilized plasmid-lipid particles (SPLP) load DNA plasmids into their aqueous core.[9,10,11] Lipoplexes[12], as well as LNPs, have been loaded with several nucleic acid cargo types including siRNA, mRNA, microRNA, and DNA.[8] A summary of the compositions of different vehicle types and exemplar lipids representing those in this study are shown in Table 1.

Table 1. Summary of the composition of different vehicle types and exemplar lipids. Lipid nanoparticle (LNP) compositions are representative for Onpattro, Moderna (Spikevax), and Pfizer (Comirnaty) vaccines.

Vehicle Type	Lipid Role	Example Lipids
Liposome	Helper lipid	Cholesterol
	Phospholipid	DSPC
	PEG-lipid	PEG-DSPE
Lipoplex	Cationic lipid	DOTMA
	Phospholipid	DSPC
SPLP	Helper lipid	Cholesterol
	Phospholipid	DSPC
	Cationic lipid	DOTAP
LNP	Ionizable lipid	MC3, ALC-315, SM102
	PEG-lipid	ALC-159, PEG-DMG
	Phospholipid	DSPC
	Helper lipid	Cholesterol

Experimental

Standards and chemicals

Lipid standards N-[2,3-(dioleoyloxy) propyl]-N,N,N-trimethylammonium (DOTMA), 1,2-dioleoyl-3-N,N,N-trimethylammonium-propane (DOTAP), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), and cholesterol were acquired from Avanti Polar Lipids, Inc. DLin-MC3-DMA (MC3), (Heptadecan-9-yl 8-((2-hydroxyethyl)(6-oxo-6-(undecyloyl) hexyl)amino) octanoate (SM-102), DMG-PEG2000, DSPE-PEG2000, 2-[(Polyethylene glycol)-2000]-N,N-ditetradecylacetamide (ALC-0159), 2-hexyl-decanoic acid, and 1,1'-[[(4-hydroxybutyl)imino]di-6,1-hexanediyl] ester (ALC 0315) were obtained from Cayman Chemical.

Each of the standards were dissolved in methanol for a stock solution and added into an equimolar mix to achieve similar peak areas. Solvents used were all LC/MS gradient grade. Ammonium acetate was obtained from Sigma-Aldrich (Steinheim, Germany).

Equipment

For the initial screening phase:

- Agilent 1260 Infinity II quaternary pump (G7111B)
- Agilent solvent selection valve: External valve drive (G1170A) + 12 pos/13 port valve (G4235A)
- Agilent 1260 Infinity II multisampler (G7116A)
- Agilent 1290 Infinity II MCT (G7116B); #058: internal valve drive
- Agilent 8-column selection valve (G4239C)
- Agilent 1290 Infinity II ELSD (G7102A)

For all other experiments:

- Agilent 1290 Infinity II bio binary pump (G7132A)
- Agilent 1290 Infinity II bio multisampler (G7137A) with Sample thermostat (option 101)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B) with the Agilent Biocompatible 6-column selector valve (part number 5320-0025) plus Biocompatible capillary kit for 6-column selector valve, 0.12 mm id (part number 5005-0070)
- Agilent 1290 Infinity II ELSD (G7102A)

Software

- ChromSwordAuto 5.1 Automated Method Development software package (version 5.1.340.944) including Chromsword AutoRobust was used for the automated experimentation
- Agilent OpenLab CDS 2.7 was used for final verification

Results and discussion

Workflow

The different chemical natures of the components shown in Table 1 make it challenging to achieve separation and good peak shapes for all components in one method. To properly evaluate the benefits of ChromSword software, the method development process contained four steps:

1. Screening (ChromSwordAuto DataSystem)
2. Optimization (ChromSwordAuto Developer/AutoRobust)
3. Robustness tests (ChromSword AutoRobust)
4. Verification (ChromSwordAuto DataSystem/Agilent OpenLab CDS 2.7)

ChromSwordAuto is commercially available and is used as standalone software to develop methods in compliance with AQbD principles. It aids automation of method development by creating all the needed acquisition methods and providing data analysis tools.

Screening

The DoE approach was implemented in this part of the study to develop a method in compliance with AQbD principles. Figure 1 shows the critical process parameters that were identified to have the greatest impact on method parameters, including selectivity, resolution, retentivity, and peak shape. Hence, the variables column chemistry, gradient time, and organic solvent composition were studied in the scouting phase of method development.

With the help of ChromSwordAuto DataSystem, which enables the use of quaternary gradient compositions, a first screening run was designed with the settings shown in Table 2. Eight gradients were applied, as shown in Figure 2. Either increasing amounts of isopropoanol as organic 1 (gradients 1 to 4) or acetonitrile as organic 1 (gradients 5 to 8) were mixed with methanol.

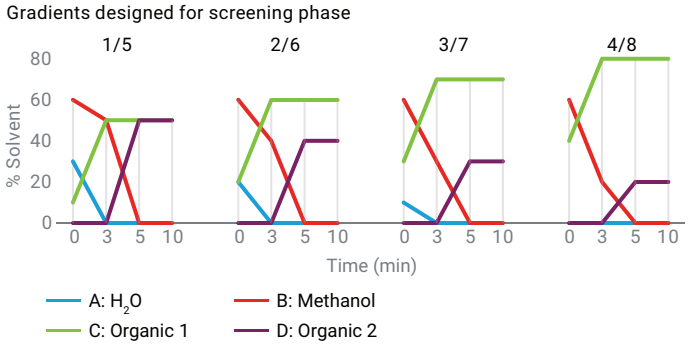


Figure 2. Gradients designed for column screening and solvent composition. Gradients 1 to 4 with an increasing percentage of isopropanol; gradients 5 to 8 with increasing percentage of acetonitrile.

Table 2. Screening parameters.

Parameter	Value
Solvents	A) 10 mM NH_4Ac in H_2O B) 10 mM NH_4Ac in 90:10 MeOH:H_2O C) 10 mM NH_4Ac in 90:10 IprOH:H_2O D) 10 mM NH_4Ac in 90:10 ACN:H_2O
Run time	10 min
Flow Rate	0.4 mL/min
Temperature	40 °C
Columns	P1: 50 × 2 mm Agilent ZORBAX Eclipse Plus Silica/C18 P2: 50 × 2 mm Agilent ZORBAX StableBond Silica/Phenyl P3: 50 × 2 mm Agilent InfinityLab Poroshell 120 PFP P4: 50 × 2 mm Agilent InfinityLab Poroshell 120 Phenyl-Hexyl P5: 50 × 2 mm Agilent InfinityLab Poroshell 120 CS-C18 P6: 50 × 2 mm Agilent ZORBAX StableBond Silica/CN

ChromSword facilitates data analysis by statistically evaluating the number of peaks per run, along with the overall resolution. As shown in Figure 3, two column/gradient combinations (ZORBAX StableBond Silica/CN and InfinityLab Poroshell 120 CS-C18) showed seven out of 10 peaks, including acceptable peak shape and best overall resolution. In general, isopropanol is the stronger solvent, and leads to less separation. Therefore, a gradient between H_2O:MeOH:ACN is preferred for the next steps. The peaks on the CS-C18 phase all eluted at the isocratic hold with 100% organic solvent. Consequently, the CS-C18 phase shows less separation power, and is considered too retentive for the target compounds selected.

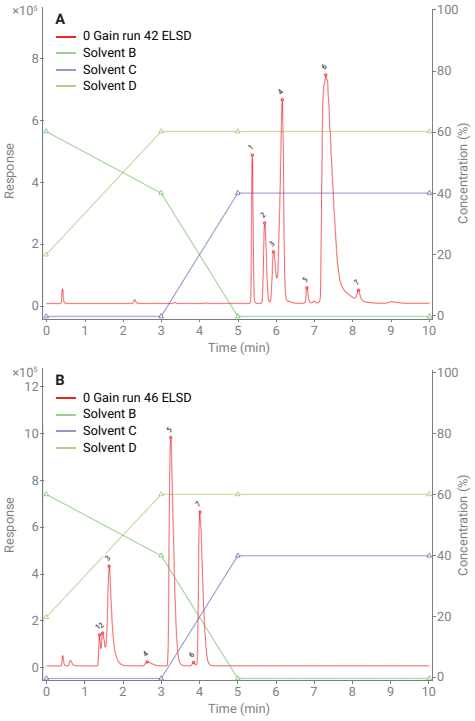


Figure 3. Results from screening. (A) Agilent InfinityLab Poroshell 120 CS-C18 with Gradient 7. (B) Agilent ZORBAX StableBond Silica/CN with gradient 7. Peak areas less than 1% were disregarded.

Optimization

Results from screening were used to design an optimization run using the algorithm "rapid optimization for large molecules" within ChromSwordAuto Developer. The 1290 Infinity II Bio LC with a binary pump was used to run fast gradients, and to minimize secondary interactions between compounds and internal surfaces. Two dimensions of the same column chemistry were chosen for this experiment:

- **P1:** 50 × 3 mm ZORBAX StableBond Silica/CN (part number 857700-305)
- **P2:** 100 × 3 mm ZORBAX StableBond Silica/CN (part number 858700-305)

Other settings were:

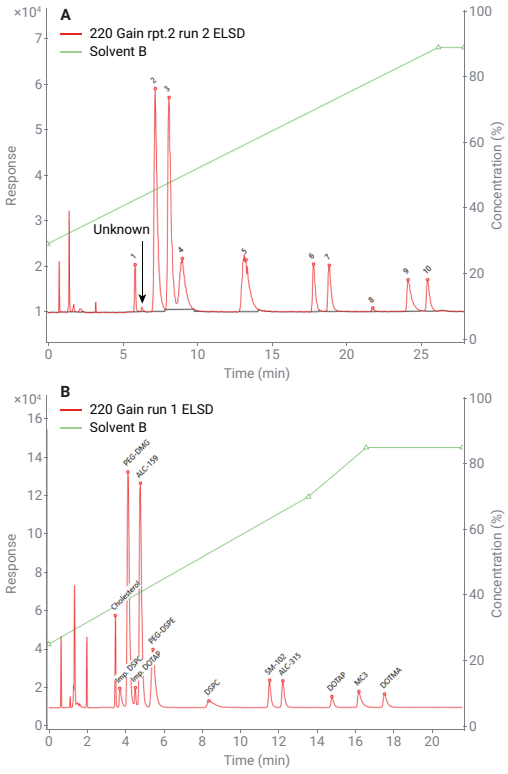
Parameter	Value
Flow Rate	0.5 mL/min
Temperature	30 °C
Run Time	Varied from 18 to 48 min
Solvent A1	10 mM NH_4Ac in 30:70 H_2O:MeOH
Solvent B1	10 mM NH_4Ac in 90:10 ACN:H_2O

Concentrations of solvents were limited inside the Developer tool to 10 to 100% of B1.

The rapid optimization algorithm designs four runs with differing gradient complexity, steepness, length, and breakpoint timing. Follow-up runs and corresponding gradients are intelligently adapted during a sequence, according to results. The results depend on the setting rejection level, which controls peak integration. Equilibration and column washout after each run are scheduled automatically.

For both column dimensions, 10 out of 10 needed peaks were detected. The 100 mm column showed superior peak shape and resolution, and the result is shown in Figure 4A.

This result is already impressive given the number of compounds and the difference in chemical properties. Interestingly, at least one extra peak moving between peak 1 and 2 was observed. This exceeded the expected number of peaks. To exclude other hidden compounds, and to account for correct peak tracking, another sequence was designed using the "rapid extended optimization for large molecules" algorithm. Similar rules apply for this algorithm, which consists of 5 to 6 runs. The concentration of solvents was limited to 25 to 85% of B1, with a flow rate set to 0.6 mL/min. The result is shown in Figure 4B.



Time (min)	A%	B%
0	75	25
13.5	30	70
16.5	15	85
21.5	15	85

Figure 4. Result optimization. (A) Best of the rapid optimization sequence with Agilent ZORBAX StableBond Silica/CN 100 mm. (B) Best of the extended rapid optimization sequence with Agilent ZORBAX StableBond Silica/CN 100 mm. Retention times less than 2 minutes were disregarded.

Results from the second round of optimization identified two underlying impurities. Careful investigation of their source through injections of single standards proved that they derive from DOTAP and DSPC. Peaks detected at less than two minutes derive from counterions of the standards used, and are disregarded for this study.

Using DoE proves useful for the detection of underlying impurities early in the method development process. Therefore, this knowledge will be used to create the MODS, and to completely understand the influence of the CPPs on method performance. Until now, the influence of flow rate and temperature had not been investigated. The basic gradient was adapted to make space for the identified impurities inside the knowledge space.

Basic method settings were:

Parameter	Value
Column	Agilent ZORBAX StableBond Silica/CN, 100 × 3 mm (p/n 858700-305)
Flow Rate	0.6 mL/min
Temperature	30 °C
Gradient	0 to 6 min 27%B 6 to 15 min 27 to 85%B 15 to 20 min 85%B
Solvent A	10 mM NH_4Ac 30:70 H_2O:MeOH
Solvent B	10 mM NH_4Ac 90:10 ACN:H_2O

Correspondingly, AutoRobust was used with the criteria shown in Table 3.

Table 3. ChromSword AutoRobust settings for design space creation.

Property	Value	±Value	±Steps
Concentration B (%)	27 start 85 end	5	3
Breakpoint Time (min)	15	1	2
Column Temperature	30	5	3
Flow Rate (mL/min)	0.6	0.1	2

Figure 5 plots temperature versus mobile phase concentration. Breaking point time variation was found to have a minor impact. The start and end concentration of solvent B, as well as temperature and flow rate, are the CPPs that have the biggest impact on peak resolution.

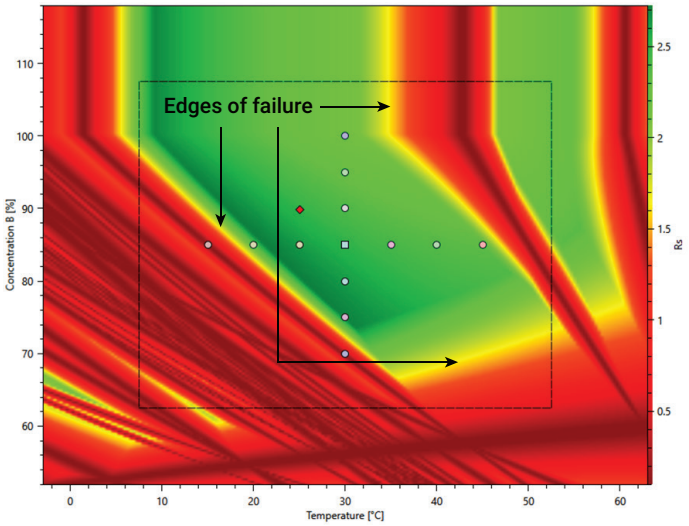


Figure 5. Method Operable Design Region (MODR) with Agilent ZORBAX StableBond Silica/CN 100 mm. Resolution set to >2. Dots represent data acquired one factor at a time; the red dot is the selected working point.

Some criteria that were chosen lead to incomplete elution of the late peaks. As such, their Retention Time (RT) prediction is inaccurate. The concerning peaks were tracked as coeluting, and are represented inside the red space of the MODR.

A working point outside the edges of failure was chosen (red dot in Figure 5). This was done, taking into consideration that the impurities will be present. In practice, the compounds will never be present together.

Working point settings were:

Parameter	Value
Column	ZORBAX StableBond Silica/CN, 100 × 3 mm (p/n 858700-305)
Flow Rate	0.5 mL/min
Temperature	25 °C
Gradient	0 to 6 min 27%B 6 to 15 min 27 to 90%B 15 to 25 min 90%B
Solvent A	10 mM NH_4Ac in 30:70 H_2O:MeOH
Solvent B	10 mM NH_4Ac in 90:10 ACN:H_2O
ELSD Settings	
Evaporator Temperature	50 °C
Nebulizer Temperature	45 °C
Gas Flow Rate	1.6 SLM
Data Rate	10 Hz

Robustness

ChromSword AutoRobust was used a second time with tighter limits, mimicking typical LC operation ranges of failure to picture the robust space. Conditions are shown in Table 4. Basic method settings are equal to the selected working point.

Table 4. ChromSword AutoRobust settings for robust space creation.

Property	Value	±Value	±Steps
Concentration B (%)	27 start 90 end	5	1
Breakpoint Time (min)	15	1	1
Column Temperature	25	5	1
Flow rate (mL/min)	0.5	0.05	1

The robust space visualization (Figure 6A) and the corresponding chromatogram (Figure 6C) verify that the working point lies comfortably inside a space in which the method is operable. When including all impurities into the robust space (Figure 6B), it becomes significantly smaller representing a worst-case scenario. Peak resolution is still >2 at the working point as shown in the chromatogram in Figure 6D. The reduction of the MODR within the worst case scenario again highlights the power of QbD. The experiment results enable method changes, such as implementation of additional occurring impurities.

Verification

The final step of Stage 1 method development is a verification of the design space. For this purpose, selected conditions inside the robust space were rerun. Results are plotted against the predicted retention times from Figure 6E. Predicted and experimental retention time match well, resulting in a coefficient of determination R^2= 0.9999.

Based on the verification data and the robustness of the method, the risk assessment indicates that there is extensive knowledge gained about the performance of the method. A suitable system suitability test may be the only control element needed in the method control strategy.

The data from the verification steps was also investigated for overall resolution inside the realistic mixtures of the different vehicle types (SPLP, lipoplex, liposomes, and LNP formulations). Overall, in all verification runs, the lowest detected resolution was 3.74. At the working point, the lowest resolution is 4.1, as shown in Figure 6F. Both were found in the composition, which equals the LNP formulation from Moderna (Spikevax). Results prove the usefulness of the method for real samples.

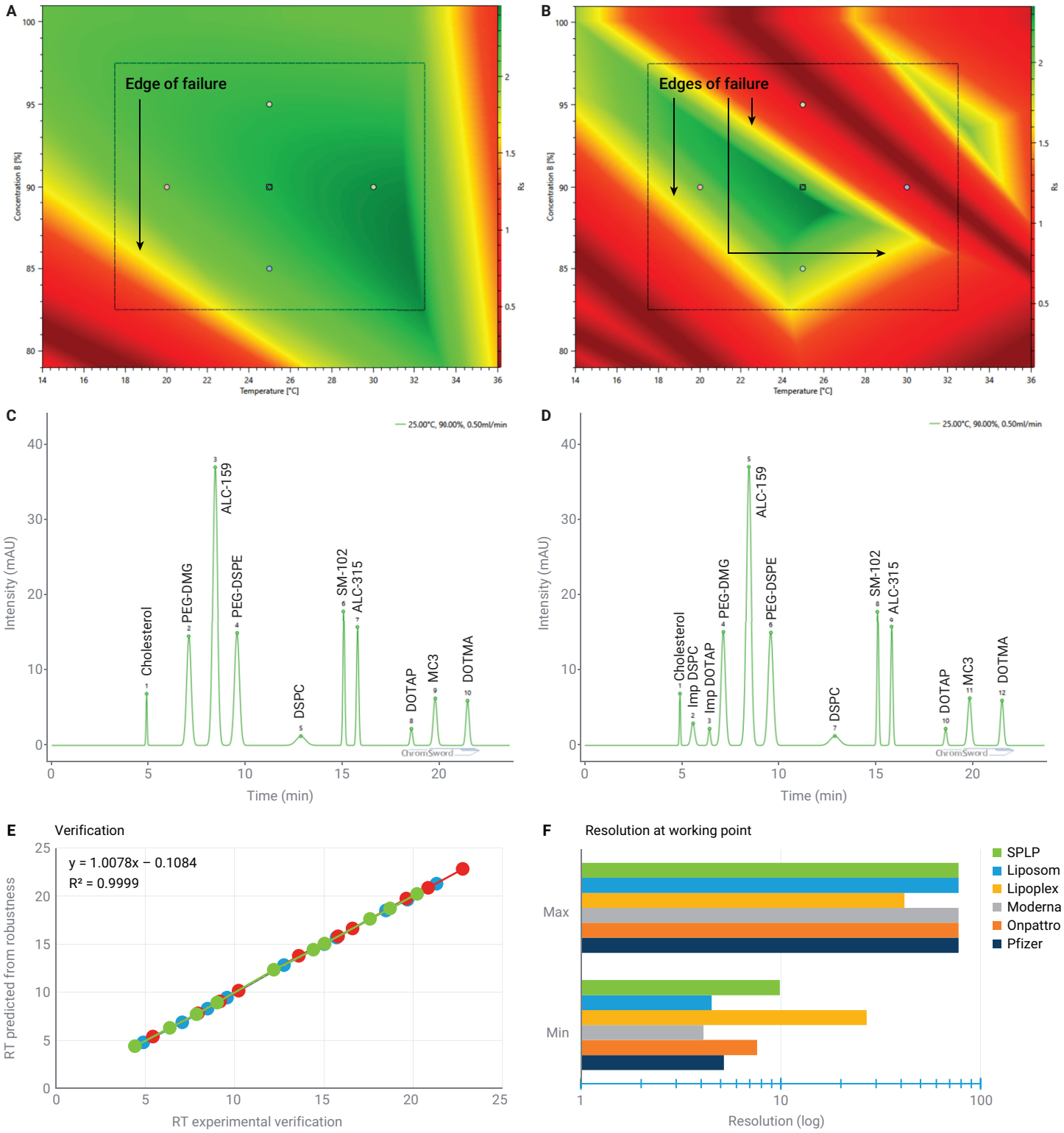


Figure 6. (A) Robust space and predicted chromatogram (C) at working point with target compounds. (B) Robust space and predicted chromatogram (D) at working point including impurities. Resolution set to >2. Dots represent data acquired one factor at a time, green dots: selected working point. (E) verification of robust space for all compounds (five verification conditions are plotted). (F) Minimum and maximum resolution for different vehicle types and lipid nanoparticle formulations at the chosen working point; X-axis in logarithmic scale.

Conclusion

The analysis of different lipid-based nucleic acid vehicle types was demonstrated using the Agilent 1290 Infinity II Bio LC and the Agilent ZORBAX StableBond Silica/CN column chemistry. Considering the challenges of modern biopharma applications with analytes of various complexities, the use of ChromSwordAuto software in conjunction with Agilent systems is beneficial for automation over the whole method development process. The efficient method development process described requires only four steps and (in total) five rounds of measurements using fit-for-purpose algorithms, incorporating the needs of the target molecules.

Using the AQbD principles in analytical method development helps to obtain robust and reproducible methods and establishes the background for further tests, including assay and purity for specific LNP formulations.

References

1. Knezevic, I. *et al.* Development of mRNA Vaccines: Scientific and Regulatory Issues. *Vaccines* **2021**, *9(2)*, 81.
2. Liu, M. A. *et al.* WHO Informal Consultation on Regulatory Considerations for Evaluation of the Quality, Safety and Efficacy of RNA-Based Prophylactic Vaccines for Infectious Diseases, 20–22 April 2021. *Emerg. Microbes & Infect*. **2022**, *11(1)*, 384-391.
3. Daniel, S. *et al* Quality by Design for Enabling RNA Platform Production Processes. Trends Biotechnol. **2022**.
4. Peraman, R.; Bhadraya, K.; Padmanabha Reddy, Y. Analytical Quality by Design: a Tool for Regulatory Flexibility and Robust Analytics. *Int. J. Anal. Chem*. **2015**.
5. Reid, G. L. *et al.* Analytical Quality by Design (AQbD) in Pharmaceutical Development. *Am. Pharm. Rev.* **2013**, 144191.
6. Schoenmaker, L. *et al.* mRNA-Lipid Nanoparticle COVID-19 Vaccines: Structure and Stability. *Int. J. Pharm.* **2021**, *601*, 120586.
7. Evers, M. J. W. *et al.* State-of-the-Art Design and Rapid-Mixing Production Techniques of Lipid Nanoparticles for Nucleic Acid Delivery. *Small Methods* **2018**, *2(9)*, 1700375.
8. Albertsen, C. H. *et al.* The Role of Lipid Components in Lipid Nanoparticles for Vaccines and Gene Therapy. *Adv. Drug Deliv. Rev.* **2022**, *188*, 114416.
9. Wheeler, J. J. *et al.* Stabilized Plasmid-Lipid Particles: Construction and Characterization. *Gene Ther.* **1999**, *6(2)*, 271–281.
10. Tam, P. *et al.* Stabilized Plasmid-Lipid Particles for Systemic Gene Therapy. *Gene Ther.* **2000**, *7(21)*, 1867–1874.
11. Maurer, N. *et al.* Spontaneous Entrapment of Polynucleotides Upon Electrostatic Interaction with Ethanol-Destabilized Cationic Liposomes. *Biophys. J.* **2001**, *80(5)*, 2310-2326.
12. Khatri, N. *et al.* Development and Characterization of siRNA Lipoplexes: Effect of Different Lipids, *In Vitro* Evaluation in Cancerous Cell Lines and *In Vivo* Toxicity Study. *AAPS PharmSciTech*. **2014**, *15*, 1630–1643.

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