Digitally Enabled Generic Analytical Framework Accelerating the Pace of Liquid Chromatography Method Development for Vaccine Adjuvant Formulations

Mohamed Hemida, Rodell C. Barrientos*, Caleb Kinsey, Nathan Kuster, Mayank Bhavsar, Armen Beck, Heather Wang, Andrew Singh, Pankaj Aggarwal, Arthur Arcinas, Malini Mukherjee, Emmanuel Appiah-Amponsah, Erik L. Regalado*

*CORRESPONDENCE:

Analytical Research and Development, MRL, Merck & Co., Inc., 126 E. Lincoln Avenue, Rahway, NJ 07065, United States of America
Phone: +1 (732) 5943789 (R.C.B.); +1 (732) 5945452 (E.L.R.). Email addresses: rodell.barrientos@merck.com; erik.regalado@merck.com

ABSTRACT: The growing use of adjuvants in the fast-paced formulation of new vaccines has created an unprecedented need for meaningful analytical assays that deliver reliable quantitative data from complex adjuvant and adjuvant-antigen mixtures. Due to their complex chemical and physical properties, method development for the separation of vaccine adjuvants is considered a highly challenging and laborious task. Reversed-phase liquid chromatography (RPLC) is among the most important tests in the (bio)pharmaceutical industry for release and stability indicating measurements including adjuvant content, identity, and purity profile. However, the time constraints of developing "on-demand" robust quantitative methods prior to each change in formulation can easily lead to sample analysis becoming a bottleneck in vaccine development. Herein a simple and efficient generic analytical framework capable of chromatographically resolving the most commonly used non-aluminum based adjuvants across academic and industrial sectors is introduced. This was designed to seek a more proactive approach for fast-paced assay development endeavors that evolved from extensive stationary phase screening in conjunction with multifactorial *in silico* simulations of adjuvant retention time (RT) as a function of gradient time, temperature, organic modifier blending, and buffer concentration. The multifactorial retention models yield 3D resolution maps with excellent baseline separation of all adjuvants in a single run, which was found to be very accurate, with differences between experimental and simulated retention times of less than 1%. The analytical framework described here also includes the introduction of a more versatile approach to method development by introducing a dynamic RT database for adjuvants covering the entire library of adjuvants with broad mechanisms of action across numerous vaccine formulations with excellent linearity, accuracy, precision, and specificity. The power of this framework was also demonstrated with numerous analytical assays that can be generated rapidly from simulations guiding vaccine processes in the development of new adjuvant formulations. Analytical assay in this work covers content, purity profile by RPLC-UV-CAD, and component identification (RPLC-MS) across complex vaccine formulations, including the use of surfactants (*e.g.*, polysorbates), as well as their separation from adjuvant targets.

Keywords: Reversed-phase liquid chromatography, vaccine adjuvant formulations, automated screening, computer-assisted optimization, charged aerosol detection.

INTRODUCTION

Recent years have witnessed an accelerated development of novel vaccine products facilitated by timely scientific research and technological innovations necessary to face global humanitarian emergencies.[1-3] Innovations in analytical methods and technologies have also played a crucial role in streamlining vaccine development. These methods enable scientists to efficiently analyze and understand the complexity of viruses, assess their pathogenicity, and identify potential targets for vaccine development.[4-7]

Characterization of antibodies, large proteins, viral vectors, virus-like particles, and other macromolecules that act as active pharmaceutical ingredients can often be challenging.[8-11] However, analytical assays that quantify small molecules present in vaccine products are needed throughout initial development to commercialization.[12-14] Among these, adjuvants are crucial components that enhance the immune response triggered by vaccines. Adjuvants boost vaccine effectiveness, enable the use of smaller vaccine doses, and potentially expand availability.[15] Typically, vaccine adjuvant formulations encompass highly diverse chemical functionalities with quite complex structures, properties, and pharmacokinetic mechanisms.[16-20]

In general, non-aluminum based adjuvant formulations are typically comprised of lipids, fatty acids, and glycosides with varying degrees of complexity and physico-chemical diversity. In addition to being diverse in nature, most vaccine adjuvants lack ultraviolet (UV) chromophores, requiring combinations of different detectors beyond traditional UV or fluorescence.[21-23] Furthermore, these molecules have a wide range of solubility and are very sensitive to pH changes.[17] Vaccine adjuvants are subjected to rigorous analytical systems for quantification, identification of degradation products, impurity profiling, and stability studies. Thus, there is a growing demand for robust and fast analytical solutions to enable both adjuvant formulation, development, and process analysis. These form the base of important quality attributes for

accelerating vaccine discovery and development. New analytical methods to address these challenges are rapidly surging in the field.[24-29]

Gas chromatography (GC) and high-performance liquid chromatography (HPLC) are commonly used separation techniques employed for lipids and adjuvants. GC can be used to characterize volatile components, whereas appropriate sample preparation is required for non-volatile components. For example, in the analysis of monophosphoryl lipid A (MPLA), a commonly used bacterial-derived adjuvant, the fatty acyl esters are first hydrolyzed and then converted to fatty acid methyl esters prior to GC analysis.[30] This method is both tedious and incapable of quantifying individual components of the mixture if prior knowledge of specific fatty acid side chains is absent.[30]

In contrast, HPLC hyphenated with widespread detectors is currently the method of choice for the analysis of lipids and adjuvants.[31-33] Reversed-phase chromatography (RPLC) remains the gold standard separation mode to resolve a wide spectrum of lipids and adjuvants based on their hydrophobicity. Mass spectrometry (MS) and evaporative light scattering detection (ELSD) are often used to analyze non-UV-absorbing adjuvants in vaccine formulations. While these detectors offer some advantages, MS is generally not the best choice for quality control (QC) laboratories under the regulation of Good Manufacturing Practices (GMP), while ELSD is known for its notoriously low detection sensitivity.

Alternatively, a charged aerosol detector (CAD) is sensitive and readily available across both early and late stages of (bio)pharmaceutical development.[34, 35] The use of HPLC-CAD has proved to bring great value for the determination of lipid content and stability, especially those that are not UV-absorbing and have low vapor pressure.[23, 33] However, there are further challenges regarding the simultaneous separation of adjuvant mixtures due to their extremely wide hydrophobicity, molecular size, and polarity range. These complex multicomponent mixtures introduce significant

analytical bottlenecks, both taxing current separation capabilities and delaying the development of new adjuvant formulations.

In recent years, analytical and synthetic chemists have partnered across the pharmaceutical and other industrial sectors to facilitate the development and validation of generic or more universal methods that cover multiple compound classes in a single experimental run. This has served to maximize the speed at which chemists can generate accurate and quality data to support the development of new synthetic routes, with immediate application in regulatory settings.[36] In spite of this, the vaccine space is yet to benefit from these advances and currently relies on the single use of LC assays. "On-demand" creation of new analytical methods is a very inconvenient, reactive approach in fast-paced laboratories, often representing a significant obstacle and source of delay (**Figure 1a**). It is evident, therefore, that a generic analytical framework that resolves the most used vaccine adjuvants in a single experimental run is missing.

Despite the great value that such a proactive approach might have across vaccine formulations, the development of new generic or more universal chromatographic methods capable of achieving baseline resolution of multiple adjuvant targets remains a tremendous challenge. Ideally, a more proactive and versatile separation concept should evolve from the integration of advanced automated stationary phase screening in conjunction with multifactorial computer-assisted modeling of adjuvant retention properties. Such a combination could yield a generic analytical framework while also enhancing method development efficiency from a dynamic built-in database that is resilient to vaccine formulation changes (**Figure 1b**).

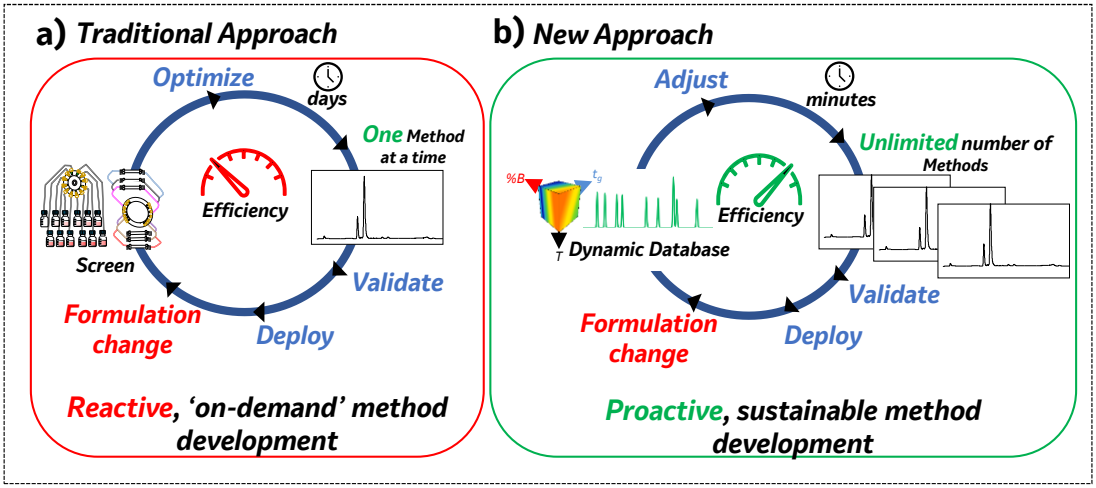

Figure 1. Current and proposed new approach for LC assay development of vaccine adjuvants.

To address these challenges, we herein introduce a digitally enabled generic analytical framework for vaccine adjuvants. With a broad selection of commonly used adjuvants in human vaccines, a dynamic RPLC database was built from *in silico* simulations of adjuvant retention time (RT) as a function of gradient time, temperature, and modifier concentration using the best column outcome of a comprehensive stationary phase and mobile phase screening. This database can be proactively used to create new methods *in silico* while bypassing resource-intensive experimentation. It is dynamic, which means that new analytes, such as novel adjuvants, excipients, or even degradants, can be added to the database on demand. To the best of the authors' knowledge, this digitally enabled generic platform assay is the first of its kind in the vaccine adjuvant space covering the widest spectrum of adjuvants used in human vaccines. The tangible power of this concept was demonstrated with real-world vaccine adjuvant formulations encountered in industry where methods that evolved from the generic analytical framework were re-adjusted *in silico* and deployed to QC laboratories.

EXPERIMENTAL

Instrumentation

Screening experiments were performed on an Agilent 1290 Infinity II UHPLC (Agilent Technologies, Santa Clara, CA), equipped with a quaternary pump (model G4204A), autosampler (model G4226A), thermostatted column compartment (model G1316C) with column selector valve (model 5067-4273), and a Diode Array Detector (DAD) module (model G7117B) with a 600 nL flow cell (model G41212-60038). The system is equipped with valve drives (model G1170A), to connect seven additional solvents to the quaternary pump. The Corona Veo RS charged aerosol detector (CAD) (Thermo Scientific, Waltham, MA) was used for the detection. High purity nitrogen was used at 60.0 psi; nebulizer temperature was 35°C, data acquisition frequency of 5 Hz; and power function was 1.

Chemicals and Reagents

Trifluoroacetic Acid (TFA), formic acid, ammonium acetate, ammonium formate, polysorbate 80 (PS-80), and triethylamine (TEA) were purchased from Sigma-Aldrich, Inc. (St Louis,MO, USA). Methanol, 2-propanol (HPLC grade) were obtained from Fisher (Fair Lawn, NJ). Acetic acid (99.7%) was purchased from Acros (West Chester, PA). Optima grade acetonitrile was purchased from Fisher Scientific (Fair Lawn, NJ). Deionized water produced from a MilliQ system (model IQ-7000) was used for mobile phase preparations. 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) (>99%), and 2-stearoyl-*sn*-glycero-3-phosphocholine (2-LysoPC), 18:0 DDAB dimethyldioctadecyl ammonium, 1-stearoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (1-LysoPC), monophosphoryl 3-deacyl lipid A (3D-PHAD®), monophosphoryl lipid A (MPLA-PHAD®), α-galactosyl ceramide (KRN7000), D-(+)-trehalose 6,6'-dibehenate (22:0 Trehalose), Squalene were obtained from Avanti Polar Lipids (Alabaster, AL). 1,2-dimyristoyl-*rac*-glycero-

3-methylpolyoxyethylene (PEG-DMG), was purchased from NOF (White Plains, NY). Cholesterol (99%) was purchased from Minakem (Dunkerque, FR). *Quillaja Saponaria* Fraction #21 (QS-21) was purchased from Desert King International (San Diego, CA).

Column and Mobile Phase Screening

Nine RPLC columns were used in the screening study (**Table 1**). All columns were used according to their manufacturer's recommendations. Different mobile phases were also screened (**Table 2**). Automated screening was enabled by column and mobile phase selector valves. All columns were operated with the same column temperature, flow rate and generic gradient: 0 – 0.5 min: 5.0% B, 0.5 –30.0 min: 5.0 - 90% B, 0.5 mL/min and 45 ℃ column temperature.

Table 1. UHPLC columns used in this study.

	Column	Dimension (I.D.) (mm) x length	Particle size (µm)	Stationary phase
1	Acquity CSH Phenyl hexyl	2.1 x 50	1.7	Phenyl hexyl
2	ZORBAX Eclipse Plus	2.1 x 50	1.8	C18
3	Acquity UPLC BEH	2.1 x 50	1.7	C18
4	InfinityLab Poroshell 120	2.1 x 50	1.9	Phenyl hexyl
5	Acquity UPLC BEH	2.1 x 50	1.7	C8
6	Acquity UPLC HSS T3	2.1 x 50	1.8	C18
7	Acquity UPLC Protein BEH	2.1 x 50	1.7	C4
8	SB-CN RRHD	2.1 x 50	1.8	CN
9	Hypersil GOLD PFP	2.1 x 50	1.8	PFP

Experimental Conditions Used for Modeling

A Waters Acquity Phenyl hexyl column was chosen for *in silico* simulation. A 3^3 experimental design was employed with column temperature (T), gradient steepness (t_G), and solvent ratio of IPA to ACN in mobile phase B as factors. Each of these factor had three levels (T = 40, 50, 60 ºC; t_G = 15, 30, 45 min; solvent ratio = 0:100, 10:90, 20:80 of ACN to IPA (*vol:vol*) as mobile phase B). The resulting experimental data were processed using the ACD Labs/LC Simulator 2021.1.2

release (Version L35R41), Advanced Chemistry Development, Inc. (ACD), Toronto, Ontario, Canada (further referred to as an LC Simulator). A resolution map was obtained from LC Simulator software, indicating the optimum separation conditions listed in **Figure 3**. The listed references describe how LC Simulator software works.[8, 37-39]

Table 2. Mobile phases used in this study

	Mobile phase
A1	0.1% Trifluoroacetic acid (TFA)
A2	0.1% Acetic acid
A3	1% Acetic acid and 0.08% of triethylamine (TEA, pH= 3.5)
A4	5 mM Ammonium formate
A5	5 mM Ammonium acetate
A6	0.1% Formic acid (FA)
B1	Acetonitrile (ACN)
B2	Methanol (MeOH)
B3	Isopropanol (IPA)

Mass Spectrometry

A Waters Xevo G2-XS Quadrupole Time-of-Flight (QToF) mass spectrometer (Waters Corp., Milford, MA) coupled with a binary Ultra-Performance Liquid Chromatography (UPLC) system was used for mass spectrometry measurements. Source conditions were as follows: Source mode: Electrospray ionization; polarity, positive and negative, mass range, 100 to 2500 *m/z*; Capillary voltage, 3.00 kV; Sampling cone, 40; Source offset, 80; Source temperature, 100 ^{o}C; Desolvation temperature, 250 ^{o}C; Cone gas, 50 L/h; Desolvation gas, 400 L/h.

Method Validation

The ICH quality guidelines for validation were followed for the validation of an assay for a four-component adjuvant formulation, as described in the results section. Attributes evaluated include the selectivity/specificity, sensitivity, linearity, precision, and accuracy of the method.

Data Analysis

All data acquisition and instrument control were performed in ChemStation C.01.07 SR3 (Agilent Technologies, Santa Clara, CA). MassLynx V4.2 (Waters Corp., Milford, MA) was used to acquire and process MS data. Multifactorial modeling of HPLC conditions was conducted in ACD/LC Simulator Software (Version L35R41) from Advanced Chemistry Development, Inc. (ACD), Toronto, Ontario, Canada.

RESULTS AND DISCUSSION

The overarching goal of this work was to create a generic analytical framework that can serve as a chromatography database of vaccine adjuvants for fast-paced method development and deployment of reliable assays. It involved the selection of a column and mobile phase combination that is suitable to resolve and elute out all the analytes. After column and mobile phase selection, a generic analytical framework with the embedded multifactorial model was built using an LC Simulator. This digitally enabled generic analytical framework can serve as a dynamic method database that can be queried rapidly to create unlimited number of assays for new vaccine formulations without the need to undergo tedious screening and optimization experiments (**Fig. 1b**).

Column and Mobile Phase Selection

Column and mobile phase selection remains a major bottleneck in developing methods for diverse pharmaceuticals.[36, 40] Lipids and adjuvants used in vaccine formulations are highly complex not only in terms of their mechanisms of action, but also in terms of hydrophobicity, molecular weight, stability, and solubility, thus posing significant analytical challenges. As such, in creating a generic analytical framework, it is important to identify the most suitable column and mobile phase at the outset.

In this work, automated UHPLC screening with multiple mobile phases and columns was employed to identify the column and mobile phase for downstream optimization.[41] A 2-position, 6-port valve was used to enable selection between nine commercial columns and nine mobile phases. The RPLC columns used in the screening exploration were carefully selected to cover a broad range of stationary phases, to comprehensively evaluate chromatographic resolution and selectivity for better study outcomes (**Table 1**). The commercial columns were selected with similar dimensions spanning diverse state-of-the-art column chemistries. Six buffers were used as aqueous mobile phases for the RPLC screening (**Table 2**). These buffers, namely ammonium formate, ammonium acetate, trifluoroacetic acid, formic acid and triethylamine were chosen because of their widespread use in adjuvant separations and compatibility with the CAD detectors.[42]

Table 3. Lipids and adjuvants used in this study

No.	Name	Abbreviation
1	*Quillaja saponaria* Fraction #21	QS-21
2	2-Stearoyl-*sn*-glycero-3-phosphocholine	18:0-LysoPC
3	1,2-Dimyristoyl-*rac*-glycero-3-methylpolyoxyethylene	PEG-DMG
4	Cholesterol	-
5	Dimethyldioctadecylammonium	DDAB
6	1,2-Dioleoyl-*sn*-glycero-3-phosphocholine	DOPC
7	1,2-Distearoyl-*sn*-glycero-3-phosphocholine	DSPC
8	Squalene	-
9	α-Galactosyl ceramide	KRN7000
10	D-(+)-trehalose 6,6'-dibehenate	22:0 Trehalose
11	Monophosphoryl 3-deacyl lipid A	3D-PHAD®
12	Monophosphoryl lipid A	MPLA/PHAD

A total of 12 common lipids and adjuvants were screened (**Table 3**). These were chosen because of their widespread use in vaccines both in commercial and clinical phase development. Some compounds are endogenous in humans (*e.g.* cholesterol, DOPC, DSPC, squalene, *etc*.), but were included here because they are commonly used to formulate adjuvants in vaccine formulations.[18]

These compounds have a wide spectrum of hydrophobicity, molecular weight, and complex chemical properties, making them extremely challenging to measure chromatographically without a systematic and holistic method development approach (**Supporting Information Table S1**).

The screening involved a total of 162 permutations resulting in data-rich results that require prompt interpretation. To streamline the decision-making process, an in-house data visualization dashboard was made to quickly identify which column provided the greatest number of peaks separated and best peak shape (**Supporting Information Fig. S1**). These results indicated that the Phenyl hexyl column delivered the best chromatographic performance for all 12 components that conveniently elute in the middle of the separation window using a standard screening gradient (**Fig. 2**). This is in contrast to C18, the most commonly used stationary phase for lipids and adjuvants[23, 43], that required a longer gradient slope at high organic composition to elute highly retained analytes (**9-12**). An overlay of chromatograms obtained using C18 and Phenyl hexyl is shown in **Fig. 2**.

The results indicate that C18 column can also be used for this work at the expense of lengthy gradient separation to ensure complete elution of hydrophobic species such as MPLA and trehalose. It is also noteworthy that using a C18 column, some of the adjuvants could co-elute that are otherwise resolved in the Phenyl hexyl column, for example, compounds **10** and **11.** This suggests that Phenyl hexyl offers additional selectivity to the hexyl phase potentially *via* pi-pi interactions induced by the phenyl ring. The extremely hydrophobic nature of a C18 stationary phase makes it a challenging column to use to build a generic analytical framework for lipids and adjuvants encountered in vaccine process development. The results also demonstrate that the use of a universal detector, CAD, is crucial as most of the lipids and adjuvants are non-UV absorbing (**Fig. 2**).

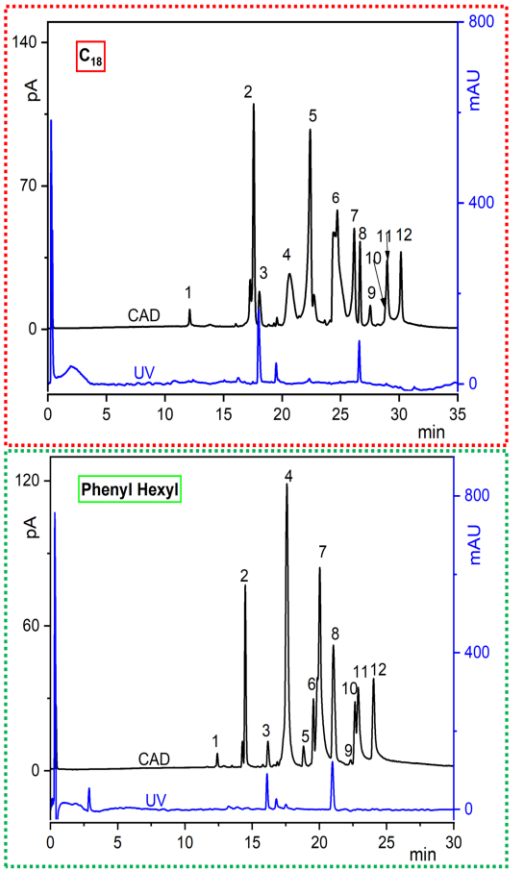

Figure 2. Chromatogram (UV 210 nm and CAD traces) obtained from method screening using generic gradient conditions. a) C18 column (1.7 µm, 4.6x50 mm I.D.) and b) Phenyl hexyl column (1.7 µm, 4.6x50 mm). Mobile phase (A: 1% acetic acid and 0.08% of triethylamine buffer (aq); B: IPA); flow rate: 0.5 mL/min; Column temperature: 40 °C. Peak assignment: (***1***) QS-21, (***2***) 18:0-LysoPC (***3***) PEG-DMG (***4***) Cholesterol, (***5***) DDAB, (***6***) DOPC, (***7***) 18:0 PC (DSPC), (***8***) Squalene, (***9***) KRN7000, (***10***) 22:0 Trehalose, (***11***) 3D-PHAD®, (***12***) MPLA/PHAD.

The screening further revealed that different mobile phase combinations can be used to construct a generic analytical framework. Previous studies have demonstrated the use of HPLC mobile phase additives such as formic acid,[22, 43] ammonium acetate,[44] ammonium formate,[45] TFA,[46, 47] acetic acid,[48] and TEA,[23] for lipids and adjuvant-related analytes. In this study, among the buffers tested (**Table 2**), 0.1% TFA, 1% acetic acid with 0.08% TEA, and 0.1% acetic acid delivered acceptable elution profiles, peak shape and resolution for the greatest number of adjuvant peaks in the mixture. Moving forward, the buffer containing 1% acetic acid with 0.08% TEA was selected for further optimization. The TEA was selected due to its volatility which makes it a good choice for CAD

detection.[23, 42] It also improves the peak shapes of cationic lipids possessing amine groups that may protonate under acidic conditions.[23] It is noteworthy that cationic lipids are becoming increasingly relevant in liposomal formulations for vaccines.[49] TEA improves the peak shape of cationic lipids by preventing secondary interactions of the amine functional groups and silica-based stationary phases.[23]

Creation of a Generic Platform Assay for Vaccine Adjuvants

The creation of a generic analytical framework involves the use of computer-assisted optimization to map the separation landscape of target adjuvants. This has been a widely used tool in developing assays that are deployed across both academic and industrial sectors.[50] Next, using the selected column and mobile phase, a suitable experimental design was conceptualized. Column temperature (T), gradient steepness (t_G), and solvent composition are the key parameters in an HPLC method that may influence the retention and resolution of a sample mixture. Using LC Simulator software, a multifactorial model was built to describe the retention behavior of the lipids and adjuvants. A total of 27 experiments (3x3x3 permutations) were used to build a 3D multifactorial model. The model consisted of three factors (T, t_G, solvent ratio) each of which had three levels (T = 40, 50, 60 ^{o}C; t_G = 15, 30, 45 min; solvent ratio = 0:100, 10:90, 20:80 of ACN to IPA (*vol:vol*)). The solvent ratio here represents the percent (volume %) of ACN in the IPA solvent used as mobile phase B. The inclusion of solvent ratio as a factor enables the versatility to optimize solvent elution strength, something that is greatly needed to tune the elution of highly retained species.

Using the experimental injections, an empirical model was constructed. **Fig. 3a** shows a snapshot of the 3D resolution map generated by the LC Simulator. The resolution map is a heatmap representation of the resolution values of critical pairs for a given chromatogram as a function of the three parameters (T, t_G, solvent ratio). The model is plotted as a spectrum of colors from blue to red to visualize the areas with the lowest and highest resolution, respectively. The desired assay condition should lie within the orange-red region of the resolution map where peaks of interest are

separated. It also shows a correlation coefficient of > 0.99, suggesting an excellent fit between the experimental and predicted retention time values. This model can be used within the bounds of the experimental limits used to construct the model (T = 40, 50, 60 °C; t_G = 15, 30, 45 min; solvent ratio = 0:100, 10:90, 20:80 of ACN to IPA (*vol:vol*). This model was tested by generating methods to separate the 12 components and experimentally verifying the retention time. Results showed that the predicted and experimental retention times matched with a highly desirable <1% retention time difference (**Fig. 3b**). This demonstrates the immense potential for the utilization of the multicolumn and multiple mobile phase screening system with software-based method development as a framework for the streamlined RPLC analysis of adjuvants in vaccine formulation development.

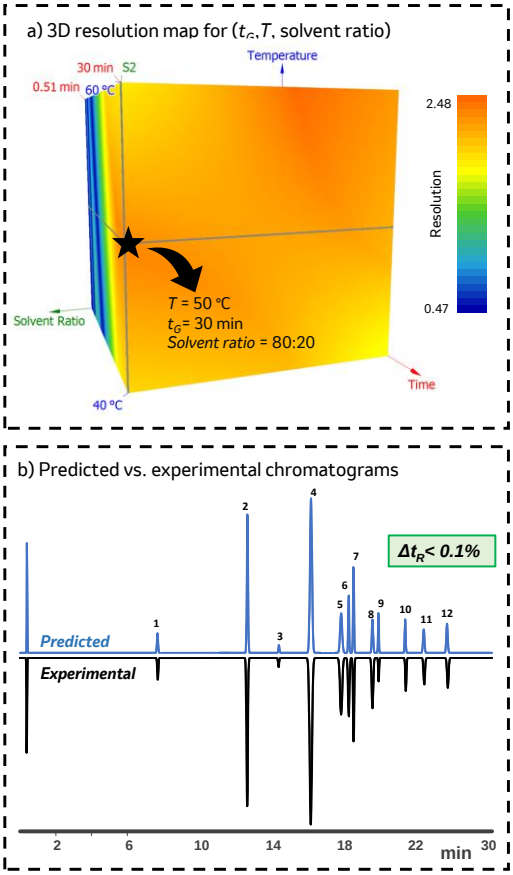

Figure 3. Multifactorial modeling to create a generic analytical framework for lipids and vaccine adjuvants. (a) Three-dimensional resolution map generated from multifactorial optimization of gradient time (t_G), column temperature (T), and solvent ratio. (b) Overlay of experimental and predicted chromatograms using conditions obtained from the model. Conditions: Phenyl hexyl

column (1.7 µm, 4.6 x 50 mm). Mobile phase (A: 1% acetic acid and 0.08% triethylamine buffer (aq); B: 80:20 IPA: ACN); flow rate: 0.5 mL/min; Column temperature: 50 ^{o}C. Peak assignment: (***1***) QS-21, (***2***) 18:0-LysoPC (3) PEG-DMG (***4***) Cholesterol, (***5***) DDAB, (***6***) DOPC, (***7***) DSPC, (***8***) Squalene, (***9***) KRN7000, (***10***) 22:0 Trehalose, (***11***) 3D-PHAD®, (***12***) MPLA/PHAD.

Evolution of Methods from the Generic Platform Assay

The goal of this work is to construct a digitally enabled generic analytical framework that can serve as a database of common lipids and adjuvants allowing the fast-paced development of methods circumventing the tedious lab-bench screening and optimization. Using this framework, new methods can be developed rapidly for any given formulation of lipids and adjuvants that are in the model. To demonstrate this, a method for a four-component adjuvant mixture (formulation **X**) was developed using the *in silico* generic framework (**Fig. 4a**). The components in this adjuvant mixture are cholesterol (**4**), DOPC (**6**), 3D-PHAD® (**11**), and MPLA (**12**), which are commercially available and used in similar ASO1b and ALFQ formulations.[17, 20, 51] This final method uses a mobile phase A of 1% acetic acid with 0.08% TEA and a mobile phase B blend of ACN and IPA at a solvent ratio of 90:10 (ACN: IPA). The column temperature of 45 ^{o}C and a runtime of 15 mins delivered baseline separation for this mixture with resolution to spare. Compared with the generic assay that is 30 mins long for all 12 adjuvants, the method for these four components was tailored to 15 mins. Because column temperature and solvent ratio are incorporated into the model, optimization of these parameters can also be performed *in silico*, thus significantly easing the experimental burden of optimizing them. Finally, this in silico-derived method was tested experimentally and confirmed that simulated and actual retention times were in close agreement (**Fig. 4a**). This showcases the versatility and value proposition that this generic analytical framework brings to the field in creating shorter RPLC assays by adjusting the conditions tailored to the analytes of interest while bypassing the lengthy and laborious experimentation.

Vaccine development is a highly dynamic process where formulations could change drastically at a fast pace. This sudden change may include the addition or removal of active components, or inactive components (excipients) in the existing formulation. In instances like this, analytical laboratories must respond promptly to develop and deploy assays for the new formulation. Developing analytical methods at this stage can be rate-limiting in the clinical path of the new formulation. Failure to develop and deploy methods swiftly can cause a significant delay in the development of a vaccine candidate.

As an example, when the four-component formulation **X** described above (Cholesterol, DOPC, 3D-PHAD®, and MPLA) underwent a sudden modification of the composition by adding a surfactant (PS-80) to yield formulation **Y** (Cholesterol, DOPC, 3D-PHAD®, MPLA and PS-80), the method previously developed was no longer suitable due to the interference of the PS-80 peaks with the target adjuvant components. PS-80 is a well-known surfactant added to vaccines and biopharmaceuticals.[52] It is a polydisperse molecule composed of fatty acid esters of polyoxyethylene sorbitan that appears as multiple peaks in RPLC, which could interfere with the target analytes.[53] Because of the addition of PS-80 to formulation **X**, a new method would be required to ensure that the lipids and adjuvant peaks are free from interference from PS-80.

Developing a method from scratch is a laborious and time-consuming process. In this work, the generic analytical framework described above was leveraged to come up with a new method for the formulation containing PS-80. Since the original model did not include PS-80, experimental injections using the same aforementioned 3x3x3 modeling conditions were performed to acquire retention time values for the PS-80 peaks. RPLC analysis of PS-80 showed five main peaks which were then incorporated into the model labeled as peaks A to E (**Fig. 4b**). This new multifactorial model was subsequently used to obtain the optimum method parameters for formulation **Y**. The new assay operates at 40 °C, a runtime of 15 mins, and a mobile phase B composition of 80:20

(ACN:IPA). The difference between predicted and experimental retention times obtained using the new model was <1% illustrating the power of modern *in silico* separation technologies.

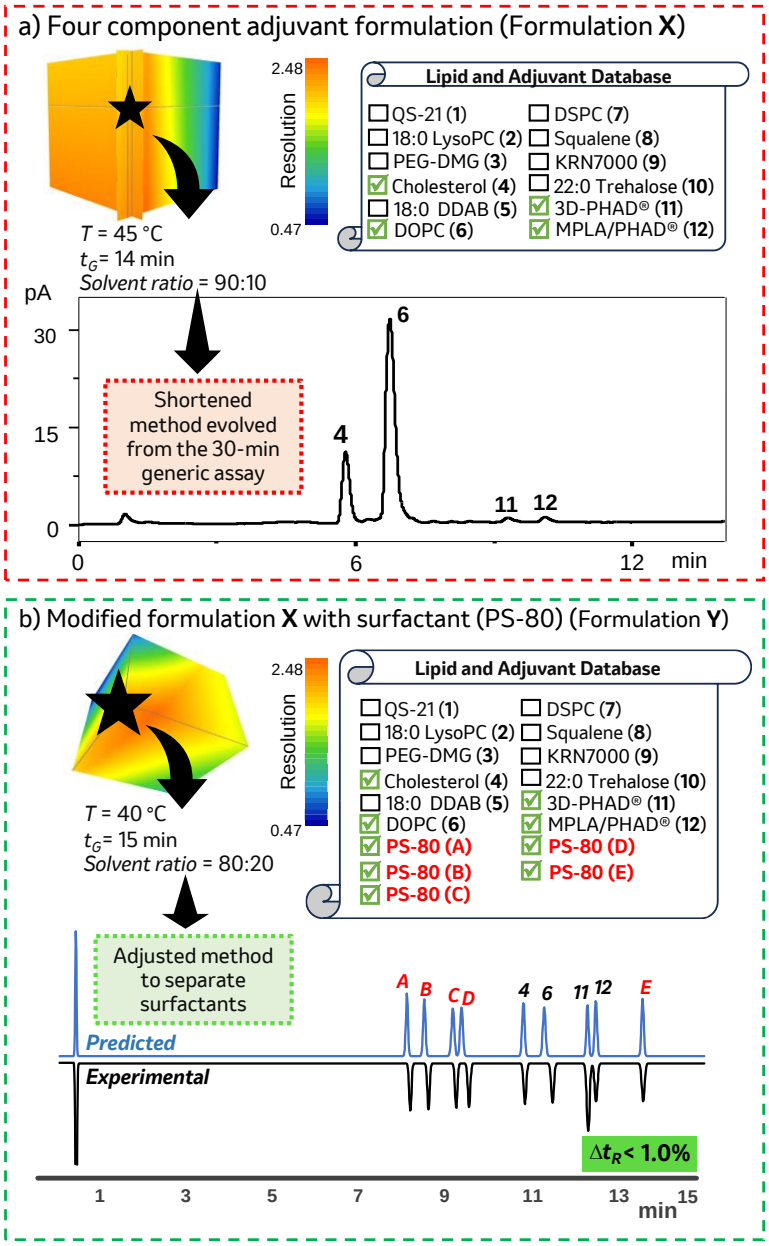

Figure 4. Development of methods leveraging the generic analytical framework. (a) 3D resolution map and chromatogram of the optimized method for formulation **X** evolved from the generic analytical framework. (b) 3D resolution map and chromatogram of the optimized method for formulation **X** modified by the addition of PS-80 (formulation **Y**). Conditions: Phenyl hexyl column (1.7 µm, 4.6x50 mm). Mobile phase (A: 1% acetic acid and 0.08% of triethylamine buffer (aq); B: 80:20 IPA: ACN); flow rate: 0.5 mL/min; Column temperature: 40 °C. Peak assignment: (***4***) Cholesterol, (***6***) DOPC, and (***12***) MPLA/PHAD.

The cases presented here demonstrate the flexibility of the model by allowing the addition of new components that result in the creation of a digital database of analytes. The same approach can be used to develop assays for novel formulations composed of different lipids, adjuvants, excipients, and even degradation products of each of the active components. In theory, this approach can yield an unlimited number of methods within minutes for any anticipated formulation change while circumventing laborious bench work and ensuring a sustainable analytical method lifecycle.

Application of the Workflow to Develop MS-Compatible Assays

One caveat of using TEA as an additive is its potential to cause persistent memory effect in mass spectrometers which makes this a less desirable MS-compatible buffer for small molecule analysis.[54] While the mobile phase described above uses a non-MS compatible buffer additive, the same experimental workflow can be used as a blueprint to construct a generic analytical framework for MS-compatible buffers. To illustrate, 10 mM ammonium acetate (*aq*) and 10 mM ammonium acetate in ACN:IPA blends were used as mobile phases A and B, respectively, to develop a model (**Fig. 5a**). To demonstrate the use of this new model, a method was developed to quantify the lipid components of an adjuvant formulation containing DOPC, Cholesterol, 3D-PHAD®, and MPLA. These adjuvant components of the formulation are outlined in order of increasing hydrophobicity as Cholesterol (**4**), DOPC (**6**), 3D-PHAD® (**11**), and MPLA (**12**) (**Fig. 5a**). Using this method hyphenated with MS, the identity of the peaks was assigned *via* electrospray ionization. MPLA as $[M-H]^-$ (*m/z* 1744.28), 3D-PHAD® was detected as $[M-H]^-$ (*m/z* 1518.08), DOPC as $[M+H]^+$ (*m/z* 786.61), and cholesterol as $[M-H_2O+H^+]$ (*m/z* 369.35) (**Figs. 5b-e**).

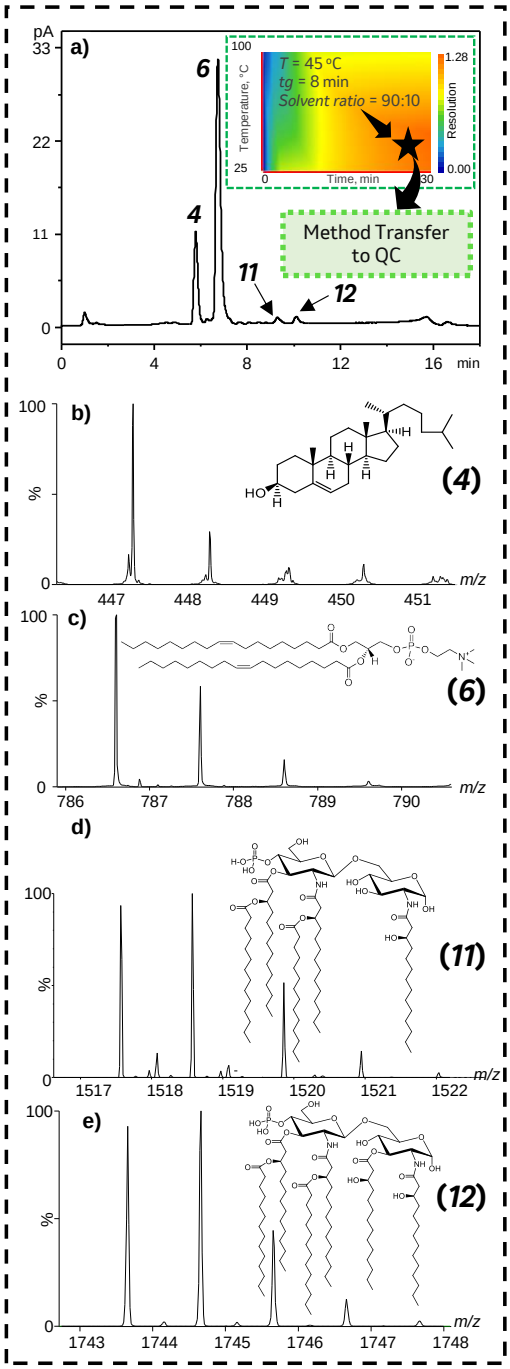

Figure 5. Development of an MS-compatible method using the generic analytical framework. (a) Resolution map and resulting chromatogram of the MS-compatible method to separate Cholesterol, DOPC, 3D-PHAD® and MPLA. (b) Mass spectrum of peak (***4***). (c) Mass spectrum of peak (***6***). (d) Mass spectrum of peak (***11***). (e) Mass spectrum of peak (***12***). Conditions: Phenyl hexyl column (2.7 µm, 3.0 x 50 mm I.D.). Mobile phase A: 10 mM ammonium acetate; Mobile phase B: 10 mM ammonium acetate in 90:10 (ACN:IPA); flow rate: 0.6 mL/min; Column temperature: 40 °C. Peak assignment: (***4***) Cholesterol, (***6***) DOPC, (***12***) 3D-PHAD® and (***12***) MPLA/PHAD.

Method Validation and Application to Vaccine Formulation Development

Important method attributes such as accuracy, linearity, dynamic range, repeatability (intra-assay precision), inter-assay precision, and specificity were all assessed for the MS-compatible UHPLC method described above and its application to vaccine adjuvant formulations. The phase-appropriate method qualification results are summarized in **Table 4**. The accuracy of this method was evaluated by standard addition of 20%, 50%, and 70% of each component in the formulation. Inter-assay precision was determined by evaluating the same sample between three separate runs on three different days. The inter-assay precision results for all lipids were between 1% and 3% (n = 6), while the intra-assay precision of eleven injections was less than 1% for all lipid adjuvant components (n = 11). Method linearity was assessed using a quadratic fitting between three independent injections within the concentration ranges (0.002 – 0.400 mg/mL). The calibration model provided acceptable linearity with a correlation coefficient (R^2) range of 0.9894-0.9998 for each quadratic fit for all the components. The method specificity evaluation revealed that the peak area of the negative control injections at the retention time of each component was < 5% of the peak area of the lowest calibration standard.

Table 4. Method qualification results for an assay to quantify a four-component adjuvant formulation

Attribute	MPLA	3D-PHAD®	DOPC	Cholesterol
Regression coefficient (R^2)	0.9940	0.9894	0.9998	0.9996
Dynamic range (mg/mL)	0.002-	0.002-0.020	0.033-0.400	0.008-0.100
Accuracy (%)	100-112	100-112	103-109	101-103
Inter-assay precision (RSD, n=6)	3%	*n/a*	1%	3%
Intra-assay precision (RSD, n=11)	1%	*n/a*	1%	1%
Specificity	Peak area of the negative control was < 5% of the lowest calibration standard			

This method was successfully applied to determine the concentration of DOPC and cholesterol in two batches of an adjuvant formulation **Z** (similar to ASO1b of GSK). Partial results of the

quantification are reported here. The target concentrations of cholesterol and DOPC were 0.50 mg/mL, and 2.000 mg/mL, respectively. In Batch 1, the concentrations measured were 2.070 mg/mL (DOPC) and 0.50 mg/mL (cholesterol). In Batch 2, the concentrations measured were 1.900 mg/mL (DOPC) and 0.46 mg/mL (cholesterol).. The precision (RSD, n = 3) was 1.1% and 3.3 % for cholesterol and DOPC, respectively. This demonstrates that methods evolved from a digitally enabled generic analytical framework can be deployed to quality control (QC) laboratories as reliable assays for batch release.

CONCLUSION

Vaccine adjuvant development is a highly dynamic process where changes in the sample formulation can happen swiftly. This often results in a need to adjust the current method or develop a completely new one. The focus of this work was to introduce a new analytical framework into the vaccine space, moving towards a paradigm shift from the traditional reactive "on-demand" assay development approach to a proactive, holistic, and sustainable way of deployment. Specifically, this work advocates for the creation of a generic analytical framework at the outset, serving as a digital library of formulation components to facilitate assay development across new vaccine processes that is resilient to adjuvant formulation changes. This framework embraces sustainability in the analytical method lifecycle by eliminating the need to perform a laborious and time-consuming column and mobile phase screening when sudden changes in the formulation take place. To this end, an automated column and mobile phase screening in conjunction with multifactorial modeling using the LC Simulator was leveraged herein to yield a generic platform assay. This approach, relying on a dynamic built-in database of retention profiles for adjuvants, excipients, or even degradants, can be leveraged for tailored RPLC-UV-CAD/MS assays that accommodate fast-paced formulation changes. The potential of this framework was demonstrated using real-world vaccine adjuvant formulations encountered in industry where methods that evolved from the generic analytical framework were re-adjusted *in silico* and deployed to QC

laboratories. The framework described here can be used not only for adjuvants but also in a wide plethora of scenarios and modalities where method adjustment is needed in response to sudden changes in sample composition. This work is a valuable contribution to the growing role of digitalization to sustainably develop and deploy new analytical assays across academic and industrial sectors.

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DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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