Development of a New Solid-Phase Extraction Base Method for Free Saccharide Content Estimation of Meningococcal Conjugate Vaccines

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ABSTRACT: GlaxoSmithKline (GSK) is currently developing a fully liquid presentation to ease the administration of the licensed quadrivalent conjugate vaccine (Menveo) against meningococcal serogroup A, C, W, and Y (MenACWY) infections. Herein, we report a new method for determining the free saccharide (FS) content of CRM_{197}-MenACWY conjugated antigens, with the aim of improving accuracy and reproducibility. Mathematical models have been used to support technical knowledge in reducing the need for experimental development. This results in an improved, faster, and platform-based technique for FS separation with one single pretreatment applicable to all antigens of the multivalent meningococcal vaccine.
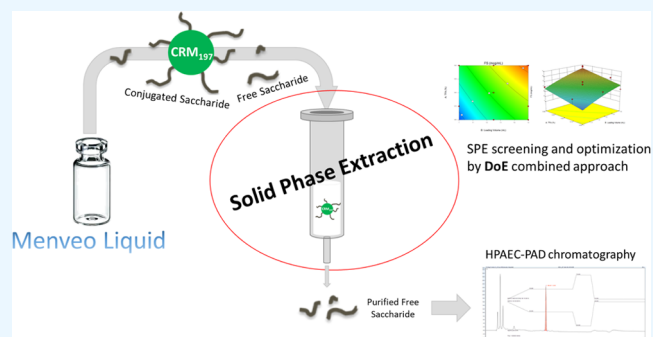
INTRODUCTION

Several glycoconjugate vaccines have been licensed or are currently in clinical development to prevent bacterial infections.[1]

The Menveo commercial vaccine (GlaxoSmithKline (GSK), formerly Novartis Vaccines - Menveo is a trademark of the GSK group of companies), which contains oligosaccharide antigens of serogroups A, C, W, and Y *Neisseria meningitidis* conjugated to a CRM_{197} protein.[2,3] It is currently available as a two-vial product, with the MenA component supplied in a lyophilized form, which is then reconstituted with the liquid MenCWY component prior to injection.[4] To simplify vaccine administration, GSK is actively working to develop the Menveo vaccine into a fully liquid, single-vial product, where all the antigens are formulated together.

The stability of the MenA polysaccharide is a major feature of the fully liquid vaccine formulation, considering its susceptibility to hydrolytic degradation in solution.[5] Therefore, accurate quantification of the free (unconjugated) saccharide (FS) is one of the most critical quality attributes to be monitored. Indeed, FS assesses the vaccine stability and integrity, determining, among other parameters, the product shelf life.[6]

A new analytical method has recently been developed with the aim of directly quantifying the glycoconjugate vaccine's active ingredients (conjugated saccharides) for the meningococcal lyophilized formulation.[7] The new method is based on the use of 30 kDa ultrafiltration to quantitatively purify the conjugated saccharide (CS) (which is collected in the retentate) from the FS (which is removed in the permeate). In this way, only the CS is measured (which is the active ingredient). This new approach has the potential to set a new route for glycoconjugate vaccine quantification, as it avoids the need for measuring both the TS and the FS amount to calculate the active ingredient concentration. Nevertheless, especially for well-established vaccine products, the measurement of FS and TS remains key to assess process consistency and to define product specifications (due to existing specification criteria that must be respected for the lifecycle of the product). For this reason, new and faster TS and FS methods have been developed and validated to support product development and process bridging with its vaccine precursor (Menveo), leveraging on previously published procedures.[8,9]

Solid-phase extraction (SPE) is a widely used technique for the purification of target analytes from complex multi-component mixtures.[10−16] SPE cartridges have already been
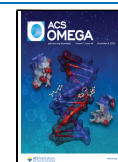
used over the last few decades in glycoconjugate-based vaccines to quantitatively separate the FS (unconjugated saccharide) from the conjugated portion (CS).[8,9] Also, alternative procedures based on FS purification via ultra-filtration procedures using various cut-off values (usually 30 kDa), have been published.[8,9,17] Particularly for published SPE procedures, C4-based cartridges were used to purify FS from CS in a reverse mode: the CS portion is not eluted and is retained in the cartridge without quantification. Following acid hydrolysis (to cleave all glycosidic bonds) and the release of the resulting monosaccharides, the purified FS is sequentially quantified by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD).

SPE cartridges for FS separation provide many advantages in routine testing, such as handling simplicity, the cleanliness of the extracted compound, and the possibility to automate the SPE purification with the use of a simple liquid handler. These advantages are of great relevance when considering the implementation of the test in commercial quality control facilities. On the other side, robust method development and the need for different elution steps are the main drawbacks of the technique.

As mentioned, the commercial Menveo vaccine contains oligosaccharide antigens of serogroups A, C, W, and Y N. meningitidis covalently conjugated to a CRM197 protein.[2,3] According to the new fully liquid MenACWY vaccine presentation, where the four antigens (same as Menveo) are formulated in the same vial in liquid solution, a method development based on quality by design (QbD) principles[18−22] in combination with a statistical approach was recently developed. This provided a procedure for the concomitant separation of all of the FS present (if at all) for each of the four glycoconjugates present within the drug product.

The obtained eluates (purified FS) are quantified via HPAEC-PAD after acid hydrolysis. As new HPAEC-PAD procedures have recently been developed to quantify each of the four meningococcal saccharides with single hydrolysis and a single HPAEC-PAD chromatography step,[17] the need for providing a universal procedure to separate and purify the unconjugated saccharides with a single pretreatment now appears more evident. The ultimate aim would be then to potentially implement the assessment of both TS and FS of all four antigens in a single run.

For these reasons, an easy, fast, and accurate nonantigen-specific FS purification method was developed. The new method explores the use of a different solid phase with respect to a classical C4 derivatized silica solid phase for the extraction of FS: the solid phase is a synthetic polymeric resin (styrene-divinylbenzene (SDB)) with an adequate pore size and sorbent mass for CRM197-conjugate retention and FS quantitative elution.

A synergistic approach toward scientific data and statistical design by applying Quality by Design (QbD) principles enabled a drastic reduction of the number of tests to be performed to screen and setup the method. The robustness of the method has been demonstrated by validating an assay that is currently implemented in quality control to release precommercial material.

■ MATERIALS AND METHODS

Drug Product. The vaccine vials containing all meningo-coccal A, C, W, and Y oligosaccharide conjugated to the carrier protein (CRM197) in liquid formulation and is produced at the GSK Manufacturing site (Italy). The final dose (0.5 mL) of MenACWY liquid vaccine contains 12, 6, 6, and 6 µg/vial each of N. meningitidis serogroup A, C, W, and Y saccharide content respectively, and formulated in 10 mM sodium phosphate buffer (pH 6.7−7.7) and 9 mg/mL of sodium chloride stored in USP Type I borosilicate glass vials.

Several MenACWY liquid development lots, produced on the bench scale, were used for the screening activities. The spike addition of representative oligosaccharides (OSs) was applied to mimic the presence of degraded materials for all serotypes to cover the developmental activity.

For method validation purposes, a MenACWY liquid Drug Product was stored at 2−8 °C for 48 months and used to explore the entire specification range for the MenA antigen. Since by simple aging it is not possible to significantly degrade MenC, MenW, and MenY antigens due to their chemical stability, for validation purposes of MenC, W and Y antigens the DP was spiked with unconjugated oligosaccharides (CWY) to cover the entire product specification range for FS.

Conditioning SPE Columns. FS quantitative determination is based on separation from the conjugated portion by solid-phase extraction cartridges (SDB-L strata 100 µm styrene-divinylbenzene 200 mg/3 mL, Phenomenex). The sample pretreatment is standardized for all serogroups and is based on three steps:

(a) SPE conditioning with methanol and water sequentially.

(b) Sample loading.

(c) Elution and recovery with a solution of acetonitrile (ACN-high-performance liquid chromatography (HPLC) grade VWR) 15% v/v and trifluoro acetic acid (TFA- Sigma Aldrich) 0.5% v/v.

Cartridge-specific conditions were developed according to specific resin properties. The conditioning of C4 cartridges for preliminary screening of all other resins: 1 mL of methanol (Carlo Erba) 100%, 2 × 1 mL of water. Loading: 1 mL of MenACWY liquid. Washing/Eluting: 1 mL of water and 3 × 1 mL of ACN 10%/TFA 0.05%. For selected cartridges, specific conditions were selected as reported in the text.

Precipitation of conjugate saccharide using DOC HCl: 5 g/L DOC (Sigma Aldrich) solution at pH 6.8 was prepared by adjusting the pH with concentrated HCl (Supelco HCl fuming 37%) approx. 1% v/v, then, 133 µL of DOC was added to 1 mL of MenACWY liquid and placed on ice for 30 minutes. Sequentially, 66.7 µL of HCl 1 N was added to the working solution and placed back on ice. Centrifugation for 15 min at 16,000g was applied and after the formation of pellet, the supernatant was recovered. The supernatant was then dried overnight, recovered in 1 mL of water and sequentially analyzed as per the standard procedure.

HPAEC-PAD Chromatographic. For MenA, MenW, and MenY quantification the cartridge's eluates are dried, reconstituted with ultrapure water, and hydrolyzed with TFA (Sigma Aldrich) 2 M for 2 h at 100 ± 2 °C to release each monomeric unit, which is analyzed by HPAEC-PAD (Thermo Fisher Scientific − ICS3000/+, ICS5000/+, ICS6000/+ models used − Chromeleon software). The external standard calibration curves are prepared from independent MenA, MenW, and MenY polysaccharide reference standards (produced and characterized by GSK) by monitoring mannos-amine-6-phosphate, galactose, and glucose, respectively. The chromatographic quantification is performed using a CarboPac
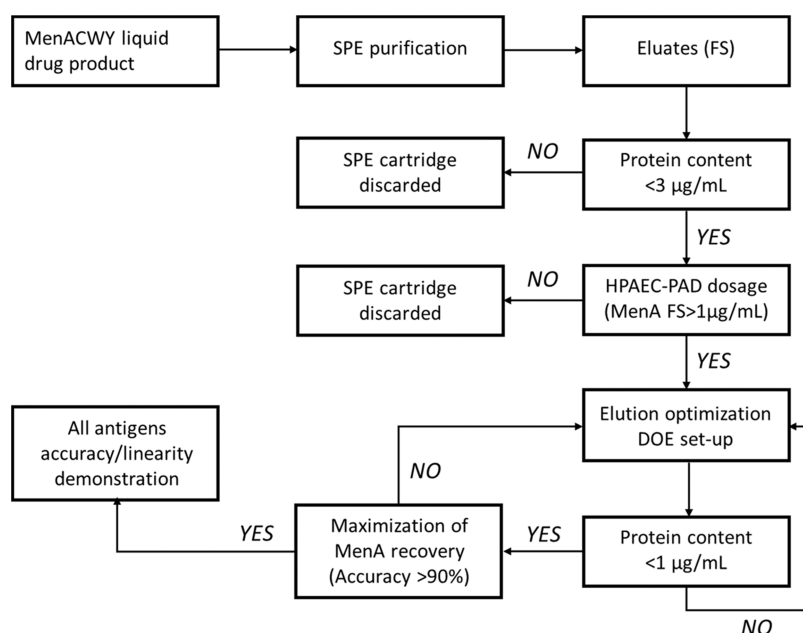
Figure 1. Workflow of SPE cartridges evaluation/optimization.

PA1 4 × 250 mm^2 column (Thermo Scientific) + CarboPac Guard Pa1 4 × 50 mm^2 (Thermo Scientific) with a flow rate of 1 mL/min. MenA is quantified in NaOH 0.1 M (JT Baker) with a gradient of sodium acetate (Thermo Scientific) 1 M (22 min run). MenW and MenY were analyzed in the same chromatography that relies on an isocratic separation in NaOH 15 mM, followed by a column cleaning in 200 mM NaOH and 1 M sodium acetate (total run time of 40 min).

For MenC, the hydrolytic conditions applied 1.2 M HCl (Supelco HCl fuming 37%) for 2.30 h at 78 °C, allow the complete hydrolysis of MenC without the release of sialic acid from MenWY.[17] The quantification in HPAEC-PAD relies on the use of MenC polysaccharide as the reference standard by monitoring sialic acid.

The chromatography separation uses a CarboPac PA1 4 × 250 mm^2 column (Thermo Scientific) + amino Trap 4 × 50 mm^2 (Thermo Scientific) with a flow of 1 mL/min. Released sialic acid is quantified in an isocratic 20 mM sodium acetate and 0.2 M NaOH chromatography (25 min).

SEC-HPLC to Assess CRM$_{197}$ Conjugate Contents in Eluates. Size-exclusion chromatography (SEC)-HPLC was performed on a Water Alliance HPLC (Empower software) using a TSKgel G3000SWXL 7.8 mm ID × 30.0 cm L Tosoh Bioscience column and a TSKgel SWXL Guard column 6.0 mm ID × 4.0 cm L Tosoh Bioscience precolumn. Ultraviolet (UV) detection at 280 nm. Elution was performed in isocratic mode with NaH$_2$PO$_4$ H$_2$O 0.1 M (Merck) + NaCl 0.1 M (Merck) pH 7.0 and using a CRM$_{197}$-bulk reference standard and calibration curves of 1, 2, 3, 6 and 9 μg/mL.

Fluorimetric Analysis. Eluates were dried and reconstituted in water (1 mL, same volume of drug product loaded into the cartridges) before being analyzed under fluorescence (Thermo Fisher Varioskan Flash Spectral Scanning Multimode Reader - SkanIT software). Excitation was set at 280 nm, and fluorescence emmission was read at 338 nm. The calibration curve was prepared starting from a concentrated CRM$_{197}$-MenA bulk produced and characterized by GSK (5 point calibration curves at 48, 24, 12, 6, and 3 μg/mL). Analysis was performed on a 96-well plate.

RESULTS

According to the different chemical properties and composition of the four oligosaccharide antigens present in the multivalent vaccine,[23] the MenA oligosaccharide is the most prone to hydrolytic degradation in a liquid formulation.[6] The instability of MenA has been linked to the presence of the phosphodiester linkage in the anomeric position of the mannosamine ring of each repeating unit, and of an axial N-acetyl decorating group in position C$_2$ that assists the breaking of the C$_1$−O$_1$ bond with subsequent removal of the phosphomonoester group. In addition, the 4-OH of N-acetylmannosamine, which is mostly not O-acetylated ($\sim$0%), can also facilitate polysaccharide hydrolysis via an intra-molecular mechanism.[5]

Therefore, FS production for MenA in liquid solution results as the main driver to assess the method performance and has consequentially been used as a pivotal antigen to evaluate the method during cartridge screening and elution development. Once the final conditions are optimized for the determination of MenA FS, the applicability to the other antigens (MenW, MenY, and MenC) was also verified.

To expand the scope of this work, for final method refinement CRM$_{197}$-MenACWY batches were thermally stressed (40 °C for at least 1 month) to increase the FS content and facilitate method development by having a significant amount of MenA FS (>1.0 μg/mL). To speed up screening, MenA-specific HPAEC-PAD chromatography was used for preliminary screening.

It is important to note that for chromatographic quantification acid hydrolysis is required to break glycosidic linkages and enable monosaccharides analysis. For this reason, during cartridge screening and optimization, the eventual presence of CRM$_{197}$-conjugates in the SPE eluates was accurately monitored: if a co-elution of FS and CS may occur, following acid hydrolysis an overestimation of FS content occurs as it is not possible to discern the monosaccharide origin. For this reason, protein content in SPE eluates was monitored using a SEC-HPLC method with a

Table 1. Summary of SPE Cartridges, Results Following Preliminary Screening, Cartridges with Acceptable Results the Regarding CRM$_{197}$ Content, FS Presence and Use of Single Cartridge are Highlighted in Gray[a]

supplier	resin type	sorbent mass/bed volume (mg/mL)	end-capped	pore size (Å)	particle size (μm)	throughput (high/medium/low)	CRM content <3.0 μg/mL	MenA (μg/mL)
Perkin Elmer	C4	100/1 (2× in series)	y	300	50	low	y	<1.0
		100/3 (2× in series)				low	y	<1.0
		200/3				medium	y	>1.0
Grace Vydac	C4	50/1 (2× in series)	y	300	13	low	y	>1.0
Phenomenex	SDB-L	100/1	N/A	260	100	high	n	>1.0
		200/3				high	y	>1.0
	polymeric strata XL	200/3	N/A	300	100	high	n	>1.0
N/A	DOC HCl	N/A				low	y	<1.0
SepaChrome	C4	100/1	y	300	13	low	y	>1.0
		200/3				medium	y	>1.0
Higgins	C4	50/1 (2× in series)	y	300	10	low	y	>1.0
		100/3 (2× in series)				low	y	<1.0
Phenomenex	phree precipitation	30	N/A			medium	y	<1.0

[a]Results were obtained by applying a general procedure for elution.

detection sensitivity of 0.1 μg/mL in carrier protein. This check avoided FS overestimation.

In summary, the two main drivers of the optimization were:

(1) To detect that CS is not present in the eluates (avoiding overestimation).

(2) To maximize the yield/recovery of FS (avoiding underestimation)

The operation workflow with relative decision points applied to the screening of the cartridges is reported in Figure 1.

As reported in Table 1, a variety of resins and formats were initially explored following the supplier guidelines for purification, roughly customizing the elution procedure to first monitor protein retention.

Generally, the procedures consisted of three main steps:

- Washing (1× column volume (CV) of MeOH and 3× CV of vaccine buffer)
- Loading of the sample (1 mL)
- Elution (3× CV of water solution containing ACN 5% TFA 0.05%)

Evaluation of CS Retention. Initially, SPE eluates were tested using fluorescence spectroscopy: CRM$_{197}$ is quantified following excitation at 280 nm and emission at 338 nm with a threshold of 3 μg/mL (using CRM-MenA standard as reference), which was considered acceptable for preliminary screening of the capacity of the resin to retain the conjugate saccharide (a 96-well plate was used).

The results of the screened cartridges are provided in Table 1. It is of note that for preliminary cartridge screening, the three elution steps mentioned above were applied, customizing if needed according to producers' guidelines. Based on spectrofluorometric results (i.e., the presence or not of glycoconjugates in the eluates) a go/no-go decision was taken for each cartridge.

For those cartridges that displayed CRM content below the selected threshold, FS quantification by HPAEC-PAD was performed.

Evaluation of MenA FS. The results obtained during the preliminary screening of SPE cartridges are reported in Table 1. Most of the cartridges confirmed the absence of CRM$_{197}$ conjugates in the corresponding eluates with a MenA saccharide quantification that roughly varies from 0.5 to 1.5

μg/mL. Cartridges with a MenA FS content below 1 μg/mL were considered not suitable and were discarded.

Although some of the tested cartridges exhibited similar performances in terms of CRM$_{197}$ retention and FS elution, a different throughput (i.e., the time required for elution) was observed amongst them. For this reason, only two SPE cartridges were finally tested for method development: a C4-based 200 mg/3 mL cartridge (Perkin Elmer) and a SDB-based cartridge 200 mg/3 mL (Phenomenex).

An improved procedure containing an additional elution step (1 water CV to remove buffer salts after sample loading) was applied to both cartridges; results are shown in Table 2.

Table 2. Summary of the CRM-MenACWY Content and MenA FS in Eluates Obtained via SDB-L and C4 Cartridges with Improved Eluting Conditions

supplier	solid phase (mg)	bed volume (mL)	CRM-MenACWY* (μg/mL)	FS (μg/mL)
Phenomenex SDB-L	200	3	<1.0	1.82
Perkin Elmer − C4	200	3	<1.0	1.47

Conditions: 1 mL of MeOH 100% and 2 × 1 mL of water; Loading: 1 mL of MenACWY liquid; washing/elution: 1 mL of water and 3 × 1 mL of ACN 10%/TFA 0.05%.

Before proceeding with cartridge development, some evaluations were conducted considering the working principle and the chemistry of the solid phase present in the two selected SPEs:

- SDB-L solid-phase extraction based on π−π binding (with aromatic amino acids present in the CRM$_{197}$ protein carrier). The phosphate group present in the MenA repeating unit is expected to be fully protonated under acidic conditions of the eluting buffer (0.5% TFA + 15% ACN) and therefore not retained by the resin.
- For C4 cartridges the interaction with the protein leverage on low energy Van der Waals (hydrophobic) interactions with amino acid containing aliphatic chains. The protein is also retained in this case, however, the possible presence of nonderivatized silanol groups (Si−

Table 3. Summary of the Risk Assessment Performed to Select the Most Critical Parameters Based on Criticality and Level of Uncertainty Following the Analytical QbD Approach[18−22]

parameters/steps	description	criticality	uncertainty	score
MeOH conditioning volume 1−3 mL	critical: MeOH is important for removing producer impurities from the resin, it also prepares/ activates the resin for separation	3	3	9
	medium−poor knowledge: a higher volume is necessary for better conditioning			
MeOH conditioning concentration (100% vs MeOH 80%)	critical: use of pure methanol or diluted methanol for conditioning the column may impact its packaging before sample loading	4	1	4
	good knowledge: it is always better to use diluted MeOH to avoid column unpacking			
water conditioning volume 1−3 mL	critical: It allows the removal of residual MeOH derived in the initial step and to condition the resin for sample loading.	4	2	8
	good knowledge: a higher volume is necessary for better conditioning.			
loading sample volume (mL)	highly critical: the sample LV is fundamental for the amount of proteins to be loaded on the resin.	5	5	25
	highly uncertain: the LV needs to be calibrated at the optimum to completely retain the protein and maximize the recovery of FS.			
washing step	noncritical: it allows the removal of the working buffer present in the sample.	2	4	8
	medium−poor knowledge: it makes the next step of separation much more efficient, poor data are available.			
organic solvent %	highly critical: it is important to correctly elute the FS.	5	3	15
	medium−poor knowledge: a minimum % of organic solvent is necessary to elute the FS, however, the exact percentage needed is not known: high % may lead to the elution of a CRM conjugate (to be avoided).			
acid concentration	highly critical: it is necessary to protonate the saccharides increasing its hydrophilicity with respect to the protein for a proper elution.	5	3	15
	medium−poor knowledge: it is not clear how much the % of organic solvent can be increased to optimize the FS elution.			
number of elution volumes	highly critical: the number of elution volumes impacts the efficiency of the separation robustness.	5	1	5
	good knowledge: from prior knowledge of SPE methods development it is well known that for this sorbent/mass ratio, a minimum of 3 mL of washing is necessary to quantitatively elute all the FS.			

OH) under acidic conditions (TFA 0.05% for C4 cartridges procedure) may reduce the efficiency in eluting the MenA FS that may weakly interact with the resin, thus reducing the recovery efficiency. It is known that although free silanol groups are inactivated by trimethylsilane (end-capping), the most rigorous end-capping procedures do not deactivate all the silanol groups present on the surface of the sorbent.[9] This results in a possible interaction between the target analyte (FS) and the resin, which may reduce the recovery efficiency.

For these reasons, the first attempt toward method optimization via a DoE statistical approach was applied using SDB-L-based cartridges.

QbD Approach for Method Optimization. Considering the operating procedure, some critical parameters/steps were identified. In a QBD approach, a risk assessment of the procedure parameters is frequently done by applying a Cause & Effect matrix. Parameters/steps were assessed as being either: noncritical, critical, or highly critical by the score, as reported in Table 3.

In this assessment, a SEC-HPLC method was set up to monitor the CRM_{197} content in cartridge eluates to reduce the limit of quantification of the protein content (CRM_{197}) from 3 to 1 μg/mL. A free CRM_{197} standard calibration was performed at the beginning and at the end of each run (first reference standard at 1 μg/mL). CRM_{197} was revealed in absorbance at 280 nm. Please note that a total protein content of 1 μg/mL, according to the degree of glycosylation, results in a negligible amount of saccharide post acid hydrolysis (<0.2 μg/mL for MenA, and lower for MenC, MenW, and MenY).

The evaluation and selection of the most relevant parameters were done by considering:

Criticality: Values from 1 to 5; 1−2 noncritical; 3−4 critical; 5 highly critical (evaluation based on the literature and producer guidelines).

Uncertainty: Values from 1 to 5; 1−2 good knowledge of the importance of the parameter; 3−4 medium−poor knowledge of the relative importance of the step; 5 high uncertainty of the relative importance of the step.

Scores: For values up to 5 the parameters are considered as not potentially critical and not relevant to be studied; from 5 to 10 the parameter is considered relatively important and will be studied with a "shootout" approach. For values above 10, the parameter is potentially critical for the development of the procedure and for process understanding and will be studied via statistical approach DoE for criticality confirmation.

In Figure 2 a schematic view of the defined procedure with the relative relevant parameters identified in the previous risk assessment is reported.

Consequentially, the less critical parameters/steps (highlighted in red in Figure 2) were explored with a shootout
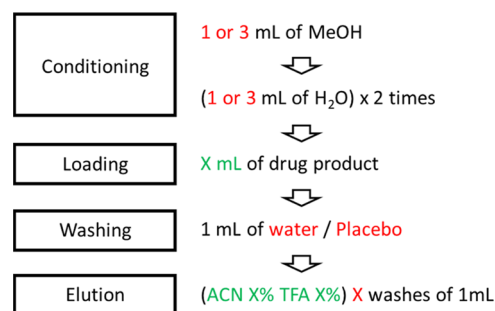
Figure 2. SPE procedural steps for the purification of FS in green parameters that are explored in a DoE and in red parameters optimized with a shootout approach.

Table 4. Results of the CRM$_{197}$ Content and MenA FS Exploring Column Conditioning and Different Washing Steps

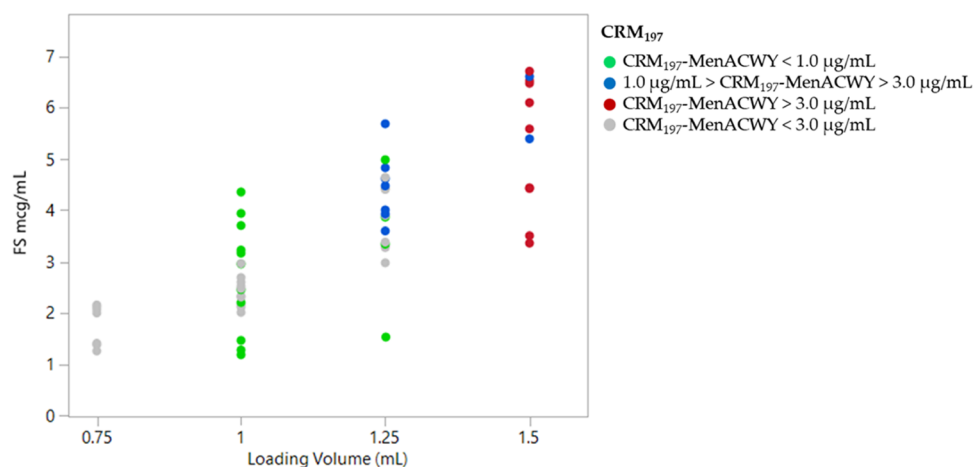| resin/volume (mg/mL)	conditioning			loading MenACWY liquid (mL)	washing (1 mL)	elution (mL)	CRM$_{197}$* (μg/mL)	FS** (μg/mL)
	MeOH (mL)	placebo (mL)	water (mL)					
200/3	1	1 (×2)	no	1	water	1 (×3)	<1.0	1.06
	1	1 (×2)	1	1	water	1 (×3)	<1.0	0.86
	3	1 (×2)	no	1	water	1 (×3)	<1.0	1.14
	3	3 (×2)	3	1	water	1 (×3)	<1.0	1.01
	3	3 (×2)	3	1	placebo	1 (×3)	<1.0	0.45

Figure 3. DoE results in terms of FS (μg/mL) with respect to the loading volume. In green are the reported eluates with a CRM$_{197}$-MenACWY content of <1.0 μg/mL, in blue eluates with a CRM$_{197}$-MenACWY content between 1.0 and 3.0 μg/mL, and in red eluates with a CRM$_{197}$-MenACWY content >3.0 μg/mL. In gray are reported eluates obtained in a preliminary DoE where the CRM$_{197}$-MenACWY content was assessed via fluorimetric analysis (LOQ of 3.0 μg/mL), and all eluates resulted in higher LOQ.
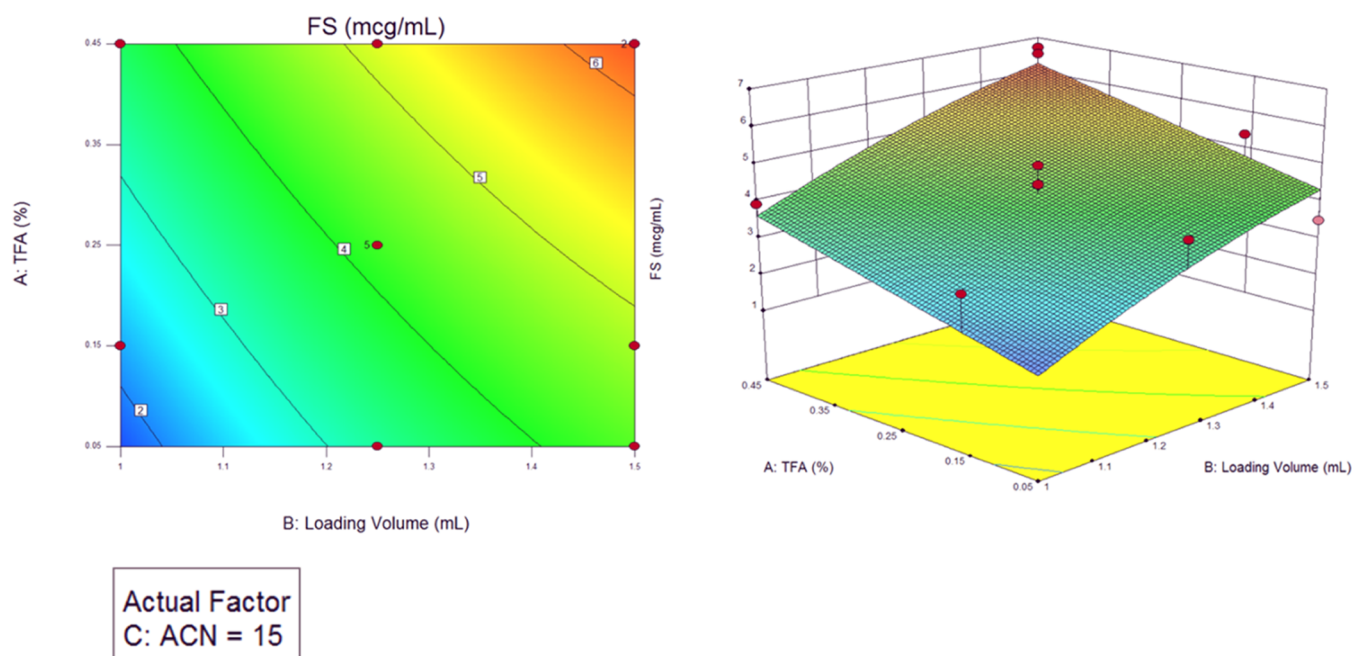

Figure 4. Response surface contour plot and 3D surface graph of MenA FS optimization DoE.

approach with the aim of maximizing FS recovery. Several settings were explored while maintaining all the critical parameters/steps fixed (loading 1 mL, ACN 10%, and TFA 0.05%) highlighted in Figure 2 in green. The results obtained within the same analytical session are reported in Table 4.

Placebo washing and conditioning consistently lead to a lower FS recovery and are consequently not suitable for this procedure. On the other hand, the addition of a water washing step allows the FS yield to increase (from 0.45 to 1.14 μg/mL, entries 3 and 5 in Table 4). Conditioning with 1 or 3 mL of MeOH and water does not appear to be critical. Thus,

considering the standard way of working recommendation (to use 5-bed volumes for complete conditioning of the resins), a conditioning volume of 3 mL was selected. The number of washing steps was investigated following the optimization of the eluting solution (ACN/TFA). For initial screening, 3 washes of 1 mL were applied.

The most critical parameters according to QbD were explored by a design of experiment (DoE) approach. To model and analyze how the elution of unconjugated saccharide, measured as FS μg/mL, is influenced, two response surface designs were conducted. The aim of the study was to identify the parameter settings able to lead to the highest level of FS μg/mL, while maintaining no detectable levels of CRM_{197}.

The design space was defined considering a loading volume range between 0.75 and 1.5 mL of MenACWY liquid (four levels), ACN range from 1 to 20% (v/v) (five levels), and TFA ranging from 0.05 to 0.45% (v/v) (five levels) as detailed below:

- Loading volume (mL): 0.75-1.0-1.25-1.50%.
- ACN: 1-5-10-15-20%TFA: 0.05-0.15-0.25-0.35-0.45.

As shown in Figure 3, loading volumes (LV) of 1.25 mL led to a small recovery of CRM_{197} conjugates under some elution conditions (between 1 and 3 μg/mL), while for an LV of 1.0 mL, no recovery of CRM_{197} conjugates was observed independently from the % of ACN and TFA. For this reason, an LV of 1 mL was selected.

Following LV lock, evaluation of the FS was considered, maximizing the FS recovery:

- TFA: 0.45% (v/v); a positive relationship was found between the levels of FS μg/mL and TFA, such that the highest level of TFA corresponds to the highest level of FS μg/mL.
- ACN: 15% (v/v); up to 5% ACN was assessed as not significant for the FS yield, while at a ratio lower than 5% a lower amount of FS was obtained.

As shown in Figure 4, higher FS values were obtained with higher amounts of TFA (0.45%), standardizing ACN at 15%.

The settings of the CPP able to deliver the highest concentration of FS μg/mL are at the edge of the design space. Therefore, optimization of the % of TFA maintaining ACN and loading volumes of 15% and 1.0 mL, respectively, was performed.

In Figure 5, TFA% of up to 0.75% was tested. The results confirmed that an increase of TFA% does not provide any benefit toward free saccharide elution, and also demonstrates the absence of any possible "in cartridge" hydrolysis effect due to the presence of TFA in the eluting buffer.

Further experiments have been performed to assess if high TFA % in the eluting solution could lead to an overestimation of the MenA FS. It was confirmed that this is not the case (see Supporting Information). For all the abovementioned reasons, 0.5% TFA was implemented.

Washing Step Robustness. As a final experiment, the number of washing steps was evaluated to check for method robustness. Up to 5 mL of eluting solution was explored (Figure 6).

No relevant increase of FS was observed for elutions from 3 to 5 mL with ACN-TFA (15%-0.5%) confirming the washing robustness, as reported in Figure 6. In addition, equivalence on FS recovery between 3 washes of 1 mL and 1 wash of 3 mL
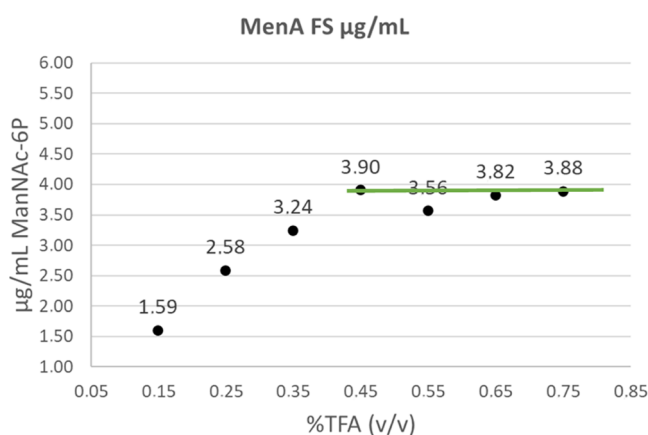
Figure 5. Evaluation of the impact of increased % TFA in the eluting solution up to 0.75% on the recovery of MenA %FS (batch #1).
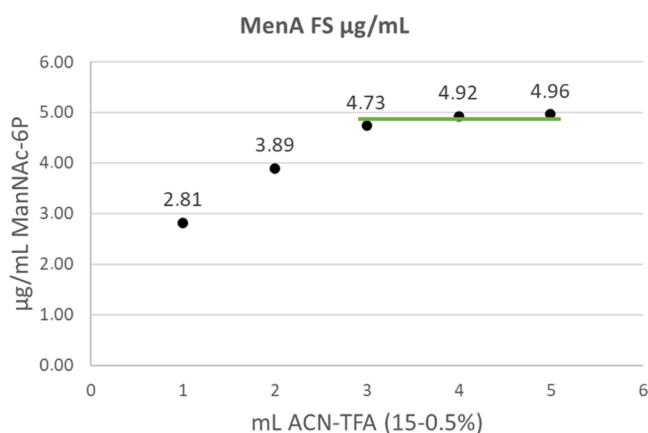
Figure 6. MenA FS amount by increasing the number of mL of the final elution step with optimized elution solution (batch #2).

was demonstrated: 1 elution of 3 mL was selected to speed up the separation timing and increase reproducibility.

Method Validation. The suitability of the procedure for all serogroups was proven and following successful method validation (reference to ICH Q2 (R1) guidance) the following parameters were evaluated and successfully met:

- Accuracy
- Intermediate precision
- Sample linearity
- Standard linearity
- Repeatability
- Specificity
- Range

In Table 5 and Table 6, a summary of data accuracy and precision is reported, demonstrating the high-performance metrics of the method. Of note is that the approach adopted for method validation regarding accuracy demonstration for MenC, MenW, and MenY explored the use of standard oligosaccharides spiked in a drug product to mimic the increasing amount of free saccharide to cover the specification range. For MenA, degraded samples with a significant concentration of MenA FS were used to cover the entire specification range (dilution approach). Results are expressed as % of recovery with respect to the theoretical amount of FS (OS titers were obtained using an orthogonal method specific for each serogroup) according to the formula reported below.

Table 5. Summary of Accuracy Validation Data Obtained for Serogroups A, C, W, and Y

	levels	MenA	MenC	MenW	MenY
accuracy (trueness)	L1	107% [CI 104; 109]	106% [CI 101; 110]	95% [CI 86; 104]	104% [CI 91; 115]
	L2	109% [CI 106; 112]	109% [CI 105; 112]	104% [CI 98; 110]	107% [CI 97; 117]
	L3	110% [CI 107; 113]	106% [CI 103; 109]	100% [CI 93; 107]	100% [CI 92; 108]
	L4	110% [CI 108; 113]	106% [CI 103; 109]	99% [CI 92; 105]	97% [CI 89; 105]
	L5	114% [CI 111, 117]	106% [CI 104, 108]	100% [CI 93, 106]	98% [CI 90, 106]
	average	110%	107%	100%	101%

Table 6. Summary of Intermediate Precision Validation Data Obtained for Serogroups A, C, W, and Y

	levels (%)	MenA (%)	MenC (%)	MenW (%)	MenY (%)
intermediate precision	L1	5	9	18	25
	L2	5	6	12	18
	L3	6	6	13	16
	L4	5	6	13	15
	L5	6	5	13	15

In addition, intermediate precision results are also reported (Table 6).

$$Recovery\% = \frac{(spiked\ DP) - (base\ level)}{theoric\ spike} * 100$$

DISCUSSION AND CONCLUSIONS

With the aim of improving the accuracy and reproducibility of the methods used for FS quantification in the MenACWY liquid formulation vaccine candidate, a detailed study on FS purification from the CRM_{197}-conjugate saccharide (that is the antigen) has been performed using SPE cartridges. Many alternative technologies around solid-phase extraction (SPE) cartridges were explored, with a few of them emerging as good candidates considering both the ability to retain the CS and the yield of FS recovery.

By considering that

(1) Synthetic resins are in general more consistent over years with respect to silica bean-derivatized resins.

(2) Synthetic resins are usually more stable over a higher pH range and over an organic solvent (used for initial conditioning and for elution).

(3) Synthetic resins are produced by many suppliers and can be produced upon request, while C4 silica-based resins can show variable performance based on the supplier (as observed in Table 1, most of the screened SPEs are C4 based but the FS amount is very different between suppliers).

Phenomenex SDB-L 200 mg/3 mL cartridges were finally selected as the ideal candidate due to their high protein retention capacity, FS recovery, and time of performance. In a QbD approach, all the potential critical factors were analyzed and screened, first with a shootout approach (for less critical attributes) then with a statistically empowered DoE. The final selected conditions consist of:

- washing with 3 mL of MeOH.
- washing with 3 mL of water (2 times).
- loading of 1 mL of MenACWY liquid.
- washing with 1 mL of water.
- 3 mL of ACN 15%−TFA 0.5%.

These conditions were finally tested for all antigens and following successful method qualification, validation was performed meeting all prefixed criteria for all antigens.

Overall, the synergy between mathematical models and technical knowledge resulted in a significant reduction in the number of experiments required to screen and develop the elution method. A fast, reproducible and robust technique for FS separation in the multivalent meningococcal vaccine was subsequently developed and validated, improving the high throughput of the assay and demonstrating high accuracy and selectivity with one single pretreatment that resulted in nonantigen dependence.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c04013.

> Additional experiments demonstrating that high TFA% in the eluting solution are not inducing MenA Conjugate Saccharide hydrolysis (PDF)

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Author Contributions

All the authors participated in the research, contributed to the writing of the manuscript, and approved the final version.

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