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Using Analytical Quality by Design to improve analytical method development in vaccines quality control: Application to an optimized quantitative high-performance anion-exchange chromatographic method

Isabelle Moineau [a], Véronique Chambon [b,*], Céline Perret [b], Luce Gouit [b], Isabelle Zamora [b], Mannaig Macumi [a], Stéphanie Fertier-Prizzon [b], Olivier Pitiot [b]

[a] AKTEHOM, Berges du Rhône, 64 avenue Leclerc, 69007 Lyon, France
[b] Analytical Sciences, Sanofi, Campus Mérieux, 1541 Avenue Marcel Mérieux, 69280 Marcy l'Etoile, France

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ABSTRACT

Analytical quality by design (AQbD) is an enhanced approach for the development of analytical methods. AQbD has received much industrial interest, being the subject of several recently published draft guidelines. This article demonstrates the application of AQbD to determine the quantity of non-adsorbed polysaccharide polyribosyl ribitol phosphate (PRP) and percentage of depolymerized PRP in a commercial hexavalent liquid vaccine, and establishment of an analytical control strategy (ACS). The quantification method developed is high-performance anion-exchange chromatography (HPAEC) with pulsed amperometric detection, preceded by ultracentrifugation (sample preparation) for separation of the depolymerized polysaccharide from the native adsorbed polysaccharide. The first step was to develop the analytical target profile (ATP) which defines the purpose of the analytical measurement as well as the development scope. As a second step, risk assessment tools were used for identification and ranking of the critical method variables (CMVs) which have a potential impact on method performance if not controlled.

Based on a multivariate Design of Experiments (DoE) approach, a proposed method operational design region (MODR) was determined for seven CMVs. Finally, the ACS was established from the understanding of the analytical method and the robustness study. This article focuses on robust and operational ranges of critical parameters linked to the ultracentrifugation and chromatographic steps for depolymerized polysaccharide content control. The design space proposed for CMVs corresponds to the ranges that ensure a product that complies with the previously established precision criteria (±2% equivalent to ± 10 % around the product criterion, which is 20 % for depolymerized polysaccharide control limit).

The following design space was established from the DoE statistical modeling for ultracentrifugation critical parameters: [483,000–520,000] g for speed, [11–19]°C for temperature, [29–34] minutes for duration, and from extemporaneous to 8 min for holding time before supernatant recuperation after the ultracentrifugation. For chromatographic critical parameters, the MODR is [2–6] psi for mobile phase helium pressure, [0–7] days for mobile phase storage time, and [0–3] days for samples storage time in the autosampler at 5 °C.

Methods optimized using the AQbD approach provide strong justifications during regulatory filing for the selection of analytical CMVs, and for the ACS to be applied during the lifecycle management of the method.

Abbreviations: ACS, Analytical Control Strategy; AQbD, Analytical Quality by Design; ATP, Analytical Target Profile; CMV, Critical Method variable; DoE, Design of Experiment; EMA, European Medicines Agency; Eur Ph, European Phamacopoeia; FMEA, Failure Modes and Effects Analysis; GMP, Good Manufacturing Practice; HPAEC, High-Performance Anion-Exchange Chromatography; HPLC, High-Performance Liquid Chromatography; ICH, International Council for Harmonisation; M, mole per liter; MHRA, Medicines and Healthcare Products Regulatory Agency; min, minute; mm, millimeter; mM, millimole per liter; MODR, Method Operable Design Region; N/A, Not Applicable; NaAc, Sodium Acetate; NaOH, Sodium Hydroxide; OOS, Out Of Specification; PAD, Pulsed Amperometric Detection; PRP, Polyribosyl Ribitol Phosphate; PRPC-T, Polyribosyl Ribitol Phosphate conjugate to tetanic toxoid protein carrier; RPN, Risk Priority Number; SST, System Suitability Test; UC, Ultracentrifugation; USP, United States Pharmacopeia.

* Corresponding author.
E-mail address: veronique.chambon@sanofi.com (V. Chambon).

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1. Introduction

Quality by design principles, based on the International Council for Harmonisation (ICH) [1–5], are largely applied to production process development in the pharmaceutical industry. Analytical methods must be viewed as a measurement process. The measurement process includes procedures, materials, critical reagents, analysts, sample preparation, environmental conditions and equipment/software. Quality risk management and statistical data analysis can be used to examine the process of measurement and identify method variables that may impact the performance characteristics of the assay (mainly specificity, precision, accuracy, linearity, detection limit and quantitation limit). Analytical quality by design (AQbD) is increasingly being applied across the pharmaceutical industry for analytical method development. Application of quality by design principles to both product/process and analytical methods development is essential to build an overall control strategy. This article presents the application of a quality by design approach to the development of an optimized analytical method (described in section 2.2) and the establishment of the analytical control strategy (ACS) [6]. This optimized method was developed to quantify the non-adsorbed polyribosyl ribitol phosphate (PRP) and determine the depolymerized PRP percentage by anion-exchange chromatography with pulsed amperometric detection in a commercial hexavalent liquid vaccine. Anion-exchange chromatography with pulsed amperometric detection is an established assay for the analysis of polysaccharide variants. However, this method is not easy to perform and presents challenges for routine use. The AQbD applied to this optimized method has permitted and reinforced the control of method critical parameters that facilities its use in routine analysis [7,8]. This innovative AQbD approach allows the identification of method parameters which have a potential impact on the method performance; the ACS can then be developed based on these critical method parameters. The approach can also simplify quality processes by reducing time for data review, training, deviation and change management by mainly considering the critical method variables (CMV). This article explains the steps, from establishing the analytical target profile (ATP [9]; defines the purpose of the analytical measurement and the development scope) to implementing the ACS by determination of the method operational design region (MODR) for CMVs. The definition of MODR will eliminate the need for regulatory re-approvals following changes in method parameters within the MODR. In addition to method development, AQbD could also be used to establish or confirm the ACS of approved methods already in routine use for commercial release in a quality control laboratory.

2. Method development

2.1. Intended use of the analytical procedure

The intended use of this optimized method is to quantify the non-adsorbed PRP content and determine the depolymerized PRP percentage by anion-exchange chromatography with pulsed amperometric detection. PRP is a linear co-polymer containing repeat units of ribosyl-ribitol-phosphate ($C_{10}H_{18}O_{11}P$) *n*. The non-adsorbed PRP content is the PRP part non– adsorbed to the aluminum gel; this represents the dose from the Haemophilus influenzae type b antigen [10]. The depolymerized PRP content is determined on the non-adsorbed PRP fraction; this is composed of free, unconjugated PRP, and also contains PRP conjugates that are comparatively smaller than the native PRP conjugate size (Fig. 1). This method is being developed to comply with both the product requirements and liquid chromatography requirements of the European Phamacopoeia (Eur Ph) [10,11,12]. The results will be used for release and stability testing of a commercial hexavalent liquid vaccine.

2.2. Analytical procedure performance characteristics

The analytical procedure characteristics, including specificity, range, accuracy, precision and limit of quantification parameters, contribute to the method performance. These must be considered during analytical procedure validation and are ultimately governed by the procedure objective, as recognized by ICH Q2 [13–19] and a consultation by the Medicines and Healthcare products Regulatory Agency (MHRA) on AQbD applied to pharmacopoeial standards for medicines [20]. Acceptance criteria for each analytical characteristic have been established, based on the release specifications and the historical validation data related to the previous chromatographic method. The analytical performance characteristics and the associated acceptance criteria for the non-adsorbed PRP, for the depolymerized PRP, and the percentage of depolymerized PRP are presented in Table 1.

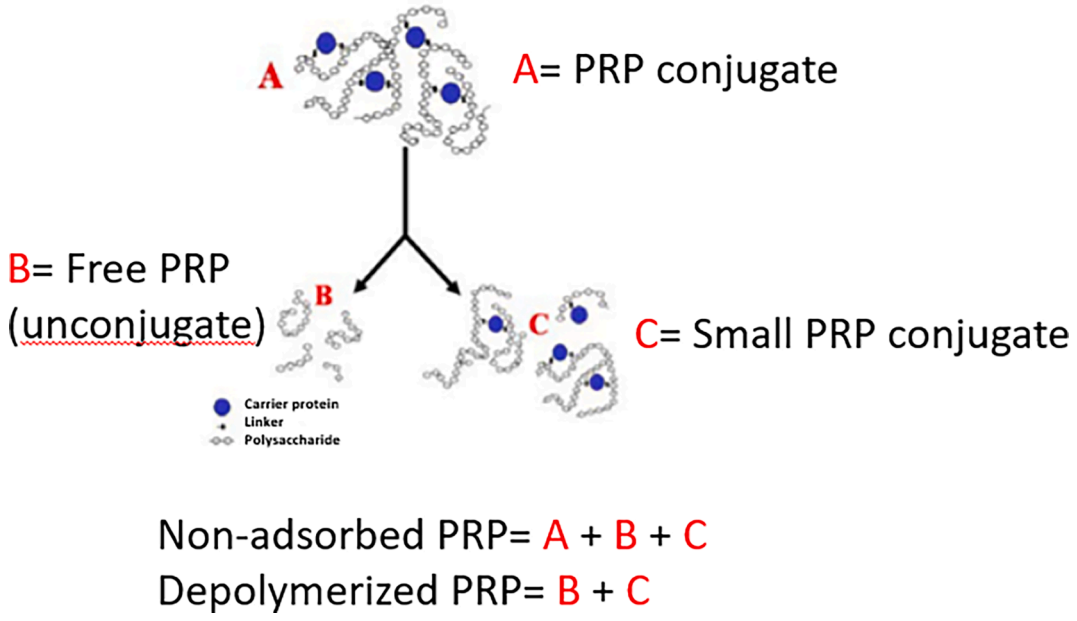

Fig. 1. Graphical illustration of the distinction between the non-adsorbed PRP content and the depolymerised PRP content.

2.3. Analytical procedure optimization

The analytical method was optimized to improve its robustness for routine analytical control. The high-performance anion-exchange chromatography (HPAEC) analysis is preceded by two separate sample preparations consisting of a low-speed centrifugation to extract the non-adsorbed PRP, and an ultracentrifugation step to separate the depolymerized polysaccharide from the native conjugated-polysaccharide. Then, both species, the non-adsorbed and the depolymerized PRP, are hydrolyzed in basic conditions into disaccharides before HPAEC-pulsed amperometric detection (PAD) analysis.

Several parameters have been optimized, with the following major improvements over the previous method:

- Use of a microbore analytical column and a gradient elution, instead of a standard dimension column and an isocratic elution, for an increased chromatographic efficiency and better detectability of products of interest [14]
- The improved method sensitivity allowed for a smaller sample quantity to be used in the test, and therefore a decrease in the sample matrix effect
- Installation of a 0.2 µm particle filter on the helium gas line upstream from the HPAEC-PAD system, with the aim of reducing trace metal that can generate organometallic complexes with phosphate moieties on the polysaccharide molecule of interest [15]

These improvements enable a more robust chromatographic method with increased precision. Furthermore, the analytical cycle time has been at least halved, with one assay instead of two independent assays, as was previously needed, to generate one sample result.

2.4. Analytical procedure conditions selected

After optimization, the analytical method conditions were selected, as described below.

Depolymerized PRP is separated from conjugated PRP by ultracentrifugation for 30 min at 12 °C and at 500,000 g and collected in the supernatant. Non-adsorbed PRP is obtained by low-speed centrifugation for 5 min at room temperature and at 5000 g to remove the aluminum gel by sedimentation. After dilution in water, the PRP from these two fractions is subsequently hydrolyzed into ribosyl-ribitol phosphate disaccharide using 1.5 M NaOH solution for 90 min at 37 °C under stirring.

The hydrolyzed samples are then analyzed by HPAEC-PAD, using an ICS-5000 (Dionex) ion chromatography system. This is equipped with a thermostated autosampler (5 °C), thermostated column compartment (30 °C), and a pulse amperometric detector (30 °C) with a conventional gold electrode. An analytical CarboPac PA10 column (2x250 mm, Dionex) is used with a guard CarboPac PA10 column (2x50 mm, Dionex). The injection volume is 15 µL. Mobile phases are composed of 100 mM NaOH/ 400 mM NaAc (solvent A) and purified water (solvent B). Separation is performed via a linear gradient program as follows: 0–5.0 min, 25 % (A); 5.0–10.0 min, linear gradient 25–50 % (A); 10.0–17.0 min, 50 % (A); 17.0–17.1 min, column regeneration 50–100 % (A); 17.1–32.0 min 100 % (A); 32.0–32.1 min, column equilibration 100–25 % (A); 32.1–42.0 min, 25 % (A) with a flow rate at 0.25 mL/min. The eluents are protected by a headspace of helium (4 psi) to prevent carbonate formation. In these chromatographic conditions, the elution time of hydrolyzate of PRP is between 12 and 14 min (Fig. 2).

The non-adsorbed and depolymerized PRP concentrations of the test samples are expressed in µg/mL relative to the reference standard, polyribosyl ribitol phosphate conjugated to tetanic toxoid protein carrier (PRPC-T), assayed under the same conditions. The percentage of depolymerized PRP is the ratio of depolymerized PRP content to non-adsorbed PRP content multiplied by 100.

3. Critical method variables

In the AQbD methodology, a risk assessment follows method optimization. This is to identify the operating conditions and the material attributes that may affect the analytical procedure performance. Various risk assessment techniques, such as Failure Modes and Effects Analysis

Table 1
Performance characteristics for determination of non-adsorbed PRP content, depolymerized PRP content, and depolymerized PRP percentage.

		Performance characteristics				
		Specificity	**Range**	**Accuracy**	**Precision**	**Limit of quantification**
Non-adsorbed PRP content determination	**Acceptance criteria**	No interfering peak appears on matrix chromatograms at PRP retention times on the non-adsorbed PRP chromatogram	10.4–29.9 µg/mL	Percent recovery included between 80 and 120 %	The 95 % CI of intermediate precision for one run with one measurement is $\leq \pm$ 2.0 µg/mL*	–
	Rationale	The observed signal must be specific of non-adsorbed PRP	Based on specification and formulated target value	Based on specification and formulated target value	The $\pm$ 2.0 µg/mL criterion corresponds to an acceptable percent of variability regarding the non-adsorbed PRP formulation target	–
Depolymerized PRP content determination	**Acceptance criteria**	No interfering peak appears on matrix chromatograms at PRP retention times on the depolymerized PRP chromatogram	1.3–13.5 µg/mL	Percent recovery included between 80 and 120 %	–	1.3 µg/mL
	Rationale	The observed signal must be specific of depolymerized PRP	Based on specification limit to monitor possible trends in production	Based on specification limit to monitor possible trends in production	–	Lower range limit verification
Depolymerized PRP percentage determination	**Acceptance criteria**	–	–	–	The 95 % CI of intermediate precision for one run with one measurement is: $\leq \pm$ 2.0 %*	–
	Rationale	–	–	–	Precision of $\pm$ 2 % for the 95 % CI of intermediate precision corresponds to an acceptable % of the release limit	–

*CI calculated using the standard deviation of intermediate precision during validation, it represents the CI for routine results.
CI, confidence interval; PRP, Polyribosyl Ribitol Phosphate.

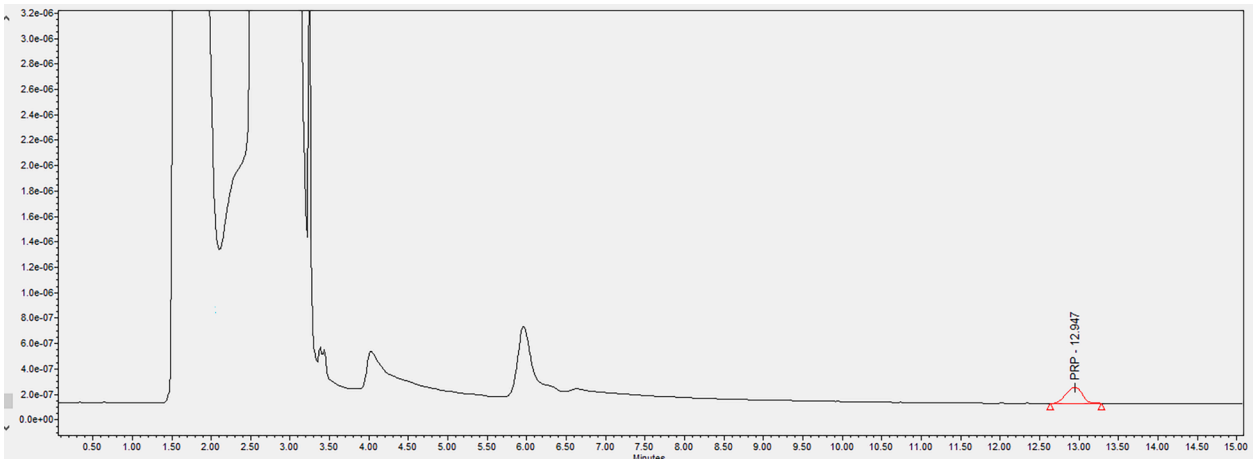

Fig. 2. Non-adsorbed PRP HPAEC chromatogram from liquid hexavalent vaccine sample.

(FMEA), may be used.

3.1. Risk identification

In our study, we used FMEA to examine specific questions of what may influence precision and/or accuracy. We mapped the proposed analytical method and applied the FMEA risk assessment to each step (Fig. 3). The optimized method includes two analytical unit operations: "Part 1" including samples and reagent preparations and "Part 2" corresponding to the chromatographic analysis (chromatographic separation and amperometric detection) (Fig. 3).

3.2. Risk evaluation and characterization

Risk assessment involves analysis of the impact of each risk on a performance attribute by characterization of the severity of this impact and by estimating the probability that this risk occurs. Scoring rules are used to determine severity and probability (Table 2a). Then the level of risk associated with a specific failure mode is evaluated using the risk priority number (RPN), which is calculated by multiplication of severity and probability (Table 2b). A method variable is identified as a critical parameter with a high-risk level, if it has a RPN score $\geq$ 25. An RPN of 25 is considered as a high risk because it corresponds to a parameter having a medium impact on the analytical procedure result, with a regular

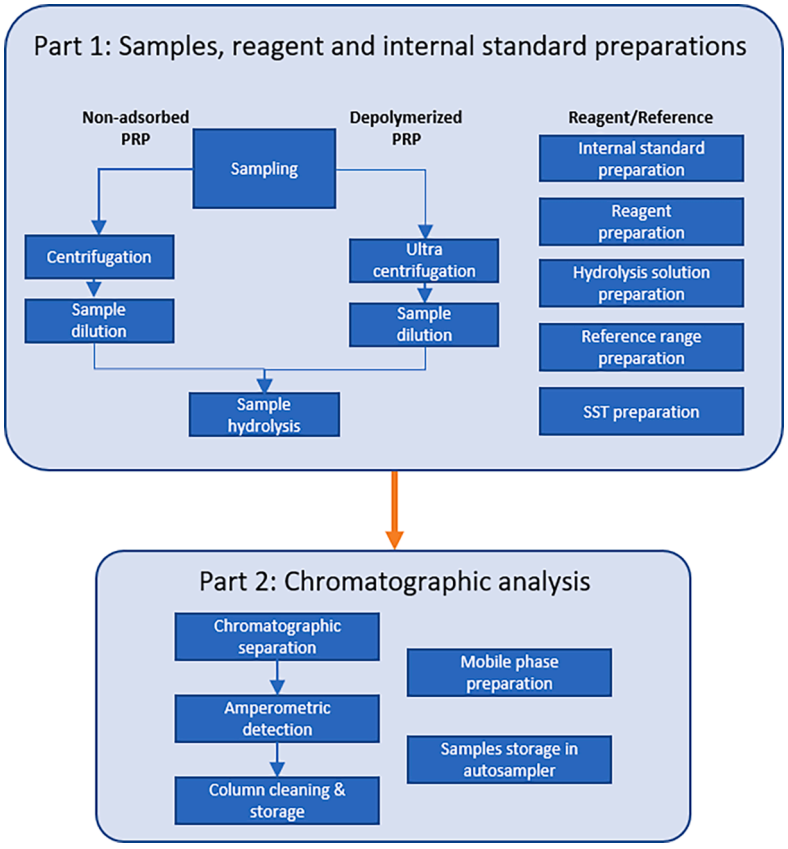

Fig. 3. Proposed analytical method: Samples, reagent and internal standard preparations (Part 1) and chromatographic analysis (Part 2) PRP, polyribosyl ribitol phosphate; SST, system suitability test.

Table 2
Risk evaluation using risk priority numbers.

Table 2a. Scoring rules used to determine severity and probability.

CRITERIA					
SEVERITY		**Score**	**PROBABILITY**		Score
No impact	No impact on the analytical procedure performance	1	**Rare**	No occurrence of failure in the life cycle of the analytical procedure	1
Medium Impact	A strong but realistic drift of the operating condition or the material attribute outside the defined intervals impacts the analytical procedure performance	5	**Possible**	One-time occurrence of failure in the life cycle of the analytical procedure	3
High Impact	A slight drift of the operating conditions or the material attribute outside the defined intervals impacts the analytical procedure performance or unknown impact (the severity was assessed as 'High Impact' when the impact of the parameter on the performance attribute is unknown)	10	**Frequent**	Regular appearance of failure in the life cycle of the analytical procedure or unknown probability (the probability was assessed as 'Frequent' when it is unknown)	5

Table 2b. Evaluation of quality risk level with Risk Priority Number.

$$Risk\ Priority\ Number = Severity \times Probability$$

Quality risk level	Risk Priority Number	Means of control
Low Risk	Score ≤ 5	Risk is acceptable – No corrective action
Medium Risk	10 ≤ Score ≤ 15	Risk to be studied case by case The lack of implementation of specific control means needs to be justified
High Risk	25 ≤ Score ≤ 50	High risk – Mandatory action Operating condition or material attribute to be controlled

appearance during the life cycle of the analytical procedure.

3.3. Risk assessment results

The initial risk assessment was used to identify non-critical parameters, critical parameters with a medium risk, and critical parameters with a high risk. For non-critical parameters, risk is considered acceptable, and no corrective actions are required. For critical parameters having a medium risk level, risk needs to be studied case-by-case and the lack of implementation of specific control means must be justified. In addition, for a parameter having a medium risk, a characterization study could be performed to improve the laboratory organization. Detailed results of the risk assessment are shown in Supplementary Data Table 1.

The risk assessment identified the CMVs having a high-risk level, for which it was mandatory to implement a means of control, and three other parameters having a medium-risk level, for which it was decided to perform a characterization study in order to optimize the laboratory organization (Table 3).

For seven method variables (Table 3), it was decided to establish a MODR using Design of Experiments (DoE) (described in the "Method Operable Design Region" section). The impact of these seven parameters on precision of the analytical method is illustrated on the fishbone diagram presented in Fig. 4. The three other parameters (Table 3) require a means of control, do not enter the robustness study, and specific corrective actions have been implemented for these (described in the "Analytical Control Strategy" section).

Table 3
Overview of risk assessment results.

Method Step	Method Variable	Risk Priority for CMV
Sample ultracentrifugation for depolymerized PRP	-Speed* -Temperature* -Time* -Holding time before supernatant sampling* -Supernatant sampling volume†	-High risk (Means of control required - MODR) -High risk (Means of control required - MODR) -High risk (Means of control required - MODR) -High risk (Means of control required - MODR) -High risk (Means of control required)
Preparation of elution mobile phase (Sodium acetate 400 mM/NaOH 100 mM)	- Mobile phase helium pressurization* - Mobile phase holding time*	-Medium (Characterization study to optimise laboratory organization - MODR) -Medium (Characterization study to optimise laboratory organization - MODR)
Chromatographic separation	-Injection volume† -Holding time of samples in the autosampler*	-High risk (Means of control required)-Medium (Characterization study to optimise laboratory organization - MODR)
Amperometric detection and integration	-Electrode type†	-High risk (Means of control required)

CMV, critical method variable; DoE, design of experiments; MODR, method operable design region; PRP, Polyribosyl Ribitol Phosphate.
*Variables for which a MODR was established using DoE.
†Parameters for which a means of control is required, which do not enter in the robustness study, and for which specific corrective actions have been implemented.

4. Method operable design region

The methodology and the data for the robustness studies for the seven selected variables are described in the following sections.

4.1. Robustness studies

DoE methodology [21] was used to study the relationship between the seven selected CMVs and their impact on the reportable result and to determine the MODR for each CMV. For each of these, the parameters, targets and limits to be studied have been defined and justified (Table 4). The operating conditions corresponding to the target have been defined during the development of the optimized method. These conditions were used in the robustness study to generate the respective reference values of the non-adsorbed PRP and of the percentage of depolymerized PRP. These tests were performed three times independently on three different days, for the robustness study. The average of these three values was used as a reference value for statistical processing. The homogeneity of this average was verified. The response variable used in the robustness study was the percentage of depolymerized PRP. This response variable was measured and statistically analyzed to assess if any of the method

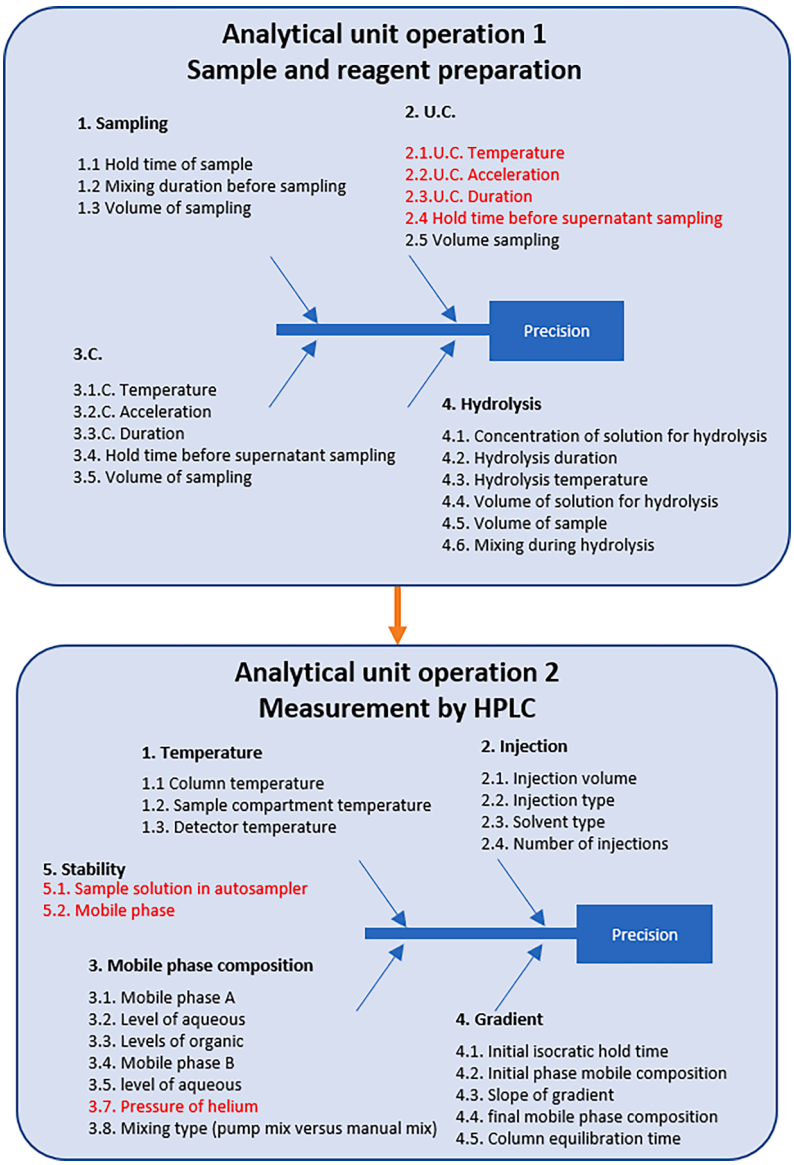

Fig. 4. Fishbone diagram showing the critical method variables identified as having an impact on precision of the analytical method (in red) CMV, critical method variable; HPLC, high-performance liquid chromatography; MODR, Method Operable Design Region; U.C., ultracentrifugation.

variables that were changed in the DoE study have any impact on this response. The evaluation of the results was carried out with regards to the measurements for determining the percentage of depolymerized PRP with respect to the reference value. Statistical data treatment which allows clear determinations regarding the significance of a variable and/or its interactions in relation to the response has been used. As a result, the study allowed the definition of the proven acceptable ranges i.e. characterized ranges of analytical parameters for which operation within this range, while keeping other parameters constant, will produce a result meeting relevant quality criteria. The quality criteria used for the statistical data treatment, for depolymerized PRP, were based on knowledge of the analytical method's precision.

The experimental scheme for the robustness study was designed with six variables which have three levels and one variable which has two levels. With this configuration, it was possible to find an orthogonal arrangement in 18 tests using the Taguchi L18 table (Table 5a), according to the method proposed by Taguchi and Konisha [16]. The Taguchi method allows efficient evaluation of each main effect independently of all others and to reveal significant interactions among the variables. The seven CMVs were varied simultaneously in a carefully planned manner, such that their individual and combined effects on the response were identified.

A specific organization of the rows of the L18 table of Taguchi has been implemented to optimize the conduct of the study in the laboratory, particularly for the pressure and the storage of the elution mobile phase (Table 5b).

4.2. Statistical data analysis

Statistical analysis was applied to depolymerized PRP data obtained from the robustness studies (Fig. 5). Observed values of the percentage of depolymerized PRP for reference assays and DoE assays are detailed in Table 6a and Table 6b respectively. Analyses relating to the percentage of depolymerized PRP for DoE assays are shown in Fig. 5 and Table 6b. The percentage of depolymerized PRP is between 9.485 % and 23.164 % for all tests conducted in the context of the robustness study, as shown in Table 6c.

4.2.1. Selection of parameters for the quantitative model

The results of the multivariate study allowed statistical modeling to

Table 4
Critical method variables and range to be studied by Design of Experiment in the robustness study.

Critical Method Variables	Range Minimum	Target	Maximum	Rationale
UC speed (g)	480,000	500,000	520,000	Based on development data and knowledge of the optimized method
UC temperature (°C)	7	12	19	
UC duration (min)	25	30	35	
Holding time before supernatant recuperation after the UC (min)	0	N/A	10	Sample handling constraint: An average time of 1 min is required to recover the supernatant after the UC and the UC rotor can contain a maximum of 10 tubes
Helium pressure of the elution mobile phase (psi)	2	4	6	Supplier's recommendations
Storage time of the elution mobile phase (day)*	D0	N/A	D + 14	Laboratory organization optimization
Storage time of samples in the autosampler at 5 °C ± 3 °C (day)†	D0	N/A	D + 3	Laboratory organization optimization

*D0 is the elution mobile phase preparation day and D + 14 is the fourteenth day after the preparation
†D0 is the sample preparation day and D + 3 is the third day after the sample preparation
D, day; UC, ultracentrifugation

obtain a complete quantitative model based on a multiple linear regression. The model takes into account the values for the parameters which have multiplier coefficients:

$$Y_i = \beta_0 + \beta_1 x_{i1} + \beta_2 x_{i2} + \beta_3 x_{i3} + \beta_4 x_{i4} + \beta_5 x_{i5} + \beta_6 x_{i6} + \beta_7 x_{i7} + \gamma_{12} x_{i1} x_{i2} + \gamma_{23} x_{i2} x_{i3} + \gamma_{13} x_{i1} x_{i3} + \gamma_{56} x_{i5} x_{i6} + \varepsilon_i$$

γ_i: response observed for depolymerized PRP (%) on test no. i, i = 1, …, i = 18.

β_0: Constant (mean effect).

β_1: Coefficient associated with the effect of ultracentrifugation speed.

β_2: Coefficient associated with the effect of ultracentrifugation temperature.

β_3: Coefficient associated with the effect of ultracentrifugation duration

β_4: Coefficient associated with the effect of the waiting time before recovery of the supernatant after UC.

β_5: Coefficient associated with the effect of the helium pressure of the mobile phase.

β_6: Coefficient associated with the effect of the storage duration of the mobile phase.

β_7: Coefficient associated with the effect of the storage duration of samples in the autosampler at 5 °C ± 3 °C.

γ_{12}: Coefficient associated with the interaction between UC Speed x UC Temperature.

γ_{23}: Coefficient associated with the interaction between UC Temperature x UC Duration

γ_{13}: Coefficient associated with the interaction between UC Speed x UC Duration

γ_{56}: Coefficient associated with the interaction between Helium pressure of the mobile phase x Storage duration of the mobile phase.

x_{i1}: Value for ultracentrifugation speed.

$x_{i\ 2}$: Value for ultracentrifugation temperature.

$x_{i\ 3}$: Value for ultracentrifugation duration

$x_{i\ 4}$: Value for waiting time before recovery of the supernatant after UC.

$x_{i\ 5}$: Value for helium pressure of the mobile phase.

$x_{i\ 6}$: Value for storage duration of the mobile phase.

$x_{i\ 7}$: Value for storage duration of samples in the autosampler at 5 °C ± 3 °C.

ε_i: Term for predictive error.

The significance of the factors and interactions was evaluated by means of a statistical test (Student) at the 5 % level. Nevertheless, a p-value between 5 and 10 %, although non-significant from a statistical perspective, is considered critical as this demonstrates a fairly high impact between the response and the variable studied. These parameters are indicated "at limit of significance" and are included in the model.

Firstly, the complete model with second order interactions was evaluated. The interactions were found not to be significant. Thus, in order to create a model which is as informative as possible, the interactions were removed in order to solely study the main effects. It corresponds to an intermediate model including the main effects only. Although the effects of ultracentrifugation speed and the waiting time before recovery of the supernatant after ultracentrifugation are at the limit of significance, the decision was made to keep them in the main effects of the model. The non-significant factors were removed from the model in order to obtain a final quantitative model with the significant effects only (Table 7).

4.2.2. Global test on the final model

The quality of the modeling was evaluated, before interpretation of the results, using a Fisher's test at the 5 % level. The objective is to verify that the estimated model is "sufficiently" informative on the variable to be explained, i.e. that the effects included have an influence on the percentage of depolymerized PRP and that an essential variable has not been overlooked.

Evaluation is conducted using Fisher's test and following hypothesis:

$\boldsymbol{H}_0 : \boldsymbol{\beta}_1 = \boldsymbol{\beta}_2 = \boldsymbol{\beta}_3 = \cdots = \boldsymbol{\gamma}_{56} = 0 \Leftrightarrow$ All coefficients are null (except the constant),

$\boldsymbol{H}_1$:There is at least one influential effect on Y out of the explanatory variables.

H_0 or H_1 will be rejected based on p value. If p value is < 0.05, hypothesis H_0 is rejected.

The F-statistic was found to be 32.97, with 4 to 13 degrees of freedom. The test showed statistical significance as p is < 0.05 (p-value $= 1.08 \times 10^{-6}$) and then hypothesis H_0 is rejected. Therefore, there is at least one influential effect on percentage of depolymerized PRP out of the explanatory variables.

R^2 represents the proportion of variability explained by the model, relative to the total variability observed. The adjusted R^2 was determined to be 88 %. This means that the model is 88 % informative, which explains nearly all the variability observed for the percentage of depolymerized PRP.

4.2.3. Evaluation of the influence of the parameters

The influence of the parameters on percentage of depolymerized PRP is shown in Table 8. Based on estimation of the parameters, the following final quantitative model was obtained: $\widehat{Y}_i$ (corresponding to the percentage of depolymerized PRP in test no. i) $= 53.9679771586851940$

$-0.0000332916402937 \times ultracentrifugation\ \ speed$

$-0.3599762996941896 \times ultracentrifugation\ \ temperature$

$-0.5537333333333334 \times ultracentrifugation\ \ duration$

Table 5

Table 5a. Taguchi L18 table for finding an orthogonal arrangement of variables, according to the method by Taguchi & Konishi [16]

	Helium pressure	Storage time of the elution mobile phase	UC speed	UC temperature	UC duration	Holding time before supernatant	Storage time of samples in the autosampler
Experiment	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1	1	1	1	1	1	1	1
2	1	2	2	3	2	2	1
3	1	3	3	2	3	3	1
4	2	1	2	2	2	3	1
5	2	2	3	1	3	1	1
6	2	3	1	3	1	2	1
7	3	1	3	3	2	1	1
8	3	2	1	2	3	2	1
9	3	3	2	1	1	3	1
10	1	1	3	2	1	2	2
11	1	2	1	1	2	3	2
12	1	3	2	3	3	1	2
13	2	1	1	3	3	3	2
14	2	2	2	2	1	1	2
15	2	3	3	1	2	2	2
16	3	1	2	1	3	2	2
17	3	2	3	3	1	3	2
18	3	3	1	2	2	1	2
	Parameter more difficult to implement						Parameter with 2 levels

Table 5b. Detailed experiments based on Taguchi L18 Table.

Row number in Taguchi L18	UC Speed (g)	UC Temp (°C)	UC duration (min)	Holding time before supernatant recuperation (min)	Helium pressure of the elution mobile phase (psi)	Storage time of the elution mobile phase (day)	Storage time of samples in the autosampler at 5°C ± 3°C (day)
7	520,000	19	30	0	6	D0	D0
16	500,000	7	35	5	6	D0	D+3
8	480,000	12	35	5	6	D+7	D0
17	520,000	19	25	10	6	D+7	D+3
9	500,000	7	25	10	6	D+14	D0
18	480,000	12	30	0	6	D+14	D+3
1	480,000	7	25	0	2	D0	D0
10	520,000	12	25	5	2	D0	D+3
2	500,000	19	30	5	2	D+7	J0
11	480,000	7	30	10	2	D+7	D+3
3	520,000	12	35	10	2	D+14	J0
12	500,000	19	35	0	2	D+14	D+3
4	500,000	12	30	10	4	D0	D0
13	480,000	19	35	10	4	D0	D+3
5	520,000	7	35	0	4	D+7	D0
14	500,000	12	25	0	4	D+7	D+3
6	480,000	19	25	5	4	D+14	D0
15	520,000	7	30	5	4	D+14	D+3

D, day; UC, ultracentrifugation

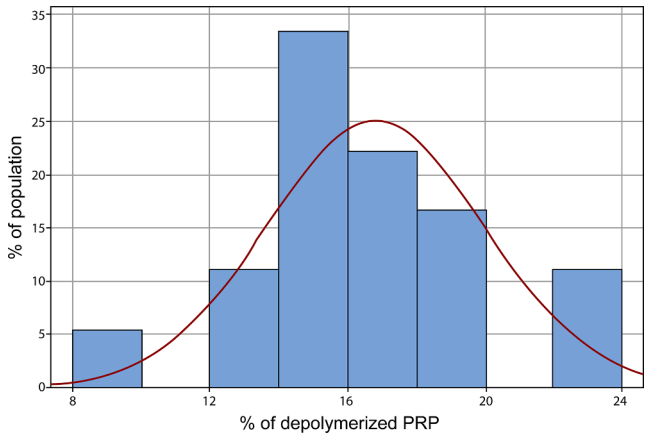

Fig. 5. Distribution of the percentage of depolymerized PRP (red line denotes a Gaussian distribution) PRP, polyribosyl ribitol phosphate.

+0.1184833333333333

× *waiting time before recovery of the supernatant after ultracentrifugation*

4.2.4. *Verification of the model at the target*

The mean value of the target tests for the percentage of depolymerized PRP re-evaluated without the "time" effect is 15.910 ± 2.0 % which corresponds to the range [13.910–17.910 %]. When including the coefficients of the final quantitative model (applied without rounding off for presentation) and the parameters at the target limits, the value estimated by the model is 16.460 %. This value estimated by the model is comparable with the mean value of the target tests, 15.910 ± 2.0 %. Consequently, the model at the target is verified.

4.3. *Determination of the acceptance range*

The estimated acceptance range is represented by the design generated by the four parameters. Defining two parameters leads to limited values for the two other parameters in order comply with the conformity of the model. In this representation, the conformity of the percentage of depolymerized PRP was defined as 15.910 ± 2.0 % which corresponds to the range [13.910–17.910 %].

Table 6

Table 6a. Observed values of percentage of depolymerized PRP for reference assays.

Target tests	% depolymerized PRP
1	15.857
2	15.977
3	15.873
4	15.914
Mean	15.910 ± 2.0 %

Table 6b. Design of Experiments assays.

Row number in Taguchi L18	% depolymerized PRP
7	14.316
16	15.577
8	15.722
17	17.496
9	23.164
18	17.746
1	22.062
10	19.505
2	15.602
11	19.788
3	13.990
12	9.485
4	15.593
13	13.890
5	15.356
14	17.847
6	17.170
15	18.245

Table 6c. Indicators for the percentage of depolymerized PRP.

Indicator	% depolymerized PRP
Mean (%)	16.808
Standard deviation (%)	3.188
Median (%)	16.446
Min (%)	9.485
Max (%)	23.164
N	18

The estimated acceptance ranges represent the values predicted by the model. The values of the acceptance ranges were rounded off, based on the currently applicable rules. These are shown in contour maps for the method variables related to the ultracentrifugation step (Fig. 6); these variables have an impact on the depolymerized PRP percentage. The estimated acceptance zone is represented by the area generated by the three parameters related to the ultracentrifugation step and the holding time before supernatant recuperation after the ultracentrifugation. The fixing of two parameters at target values leads to limited values for the two other parameters, respecting the conformity of the model. Based on the statistical results, the holding time before supernatant recuperation after the ultracentrifugation parameter was set up at eight minutes to give more flexibility for the MODR of the three other ultracentrifugation parameters. For each ultracentrifugation parameter studied, a robustness range was defined (Table 9). For the other parameters (helium pressure and stability of the elution mobile phase and stability of samples in the autosampler), statistical results showed no impact on the performance of the method within the studied range. Therefore, for these parameters, the MODR corresponds to the studied range in the robustness study.

5. Analytical control strategy

5.1. Control of critical method variables with high risk

For the CMV "supernatant sampling" at the ultracentrifugation step, a video showing the movement will be implemented as part of training before use of the optimized method in the Quality Control Laboratory. The CMV "reference electrode" and its batch-to-batch variability was not studied. Adequate performance of the electrode will be verified by the System Suitability Test (SST) in place in the method to ensure performance of the procedure; therefore there was no need for it to be studied further. The CMV "injection volume" will be controlled by a duplicate injection of each sample preparation with an acceptance criterion between duplicate values. The four remaining CMVs related to ultracentrifugation (speed, temperature, duration and holding time of

Table 7
Quantitative model with the significant effects only.

Factor	Mean square	Degrees of freedom	F statistic	*p*-value	Significance at the 5 % level
Ultracentrifugation speed (g)	4.608	1	3.86	0.071	Limit of significance
Ultracentrifugation temperature (°C)	56.498	1	47.36	1.12×10^{-5}	Significant
Ultracentrifugation duration (min)	91.986	1	77.11	7.95×10^{-7}	Significant
Waiting time before recovery of the supernatant after ultracentrifugation (min)	4.211	1	3.53	0.083	Limit of significance
Residual error	1.193	13	N/A	N/A	N/A

N/A, not assessed.

Table 8
Influence of the parameters on percentage of depolymerized PRP.

Factor	Coefficient	Impact on depo. PRP	Standard error	T value	*p*-value	Significance at the 5 % level
Constant	53.97	N/A	8.68	6.22	3.13×10^{-5}	N/A
Ultracentrifugation speed (g)	−0.000033	↘	0.000017	−1.97	0.071	Limit of significance
Ultracentrifugation temperature (°C)	−0.3600	↘	0.0523	−6.88	1.12×10^{-5}	Significant
Ultracentrifugation duration (min)	−0.5537	↘	0.0631	−8.78	7.95×10^{-7}	Significant
Waiting time before recovery of the supernatant after ultracentrifugation (min)	0.1185	↗	0.631	1.88	0.083	Limit of significance

N/A, not assessed; PRP, polyribosyl ribitol phosphate.

supernatant sampling after ultracentrifugation) and having a high level of risk were evaluated using a DoE study, and MODR was determined where the quality requirement of the reportable result established was met (Table 8).

5.2. Control of critical method variables with low/medium risk

All other CMVs, evaluated with a low or medium level of risk, were considered acceptable as they are controlled under good manufacturing practice (GMP) conditions, which assumes the use of trained personnel, as well as qualification and maintenance of equipment and operating conditions, as clearly described in the analytical procedure. However, for laboratory organization optimization, acceptable ranges were also determined for three CMVs having a medium risk level (helium pressure of the elution mobile phase, holding time of the elution mobile phase, holding time of samples in the autosampler). Results are shown in Table 9.

6. Conclusion

This article describes an AQbD approach to developing an analytical method for determining the quantity of non-absorbed polysaccharide and percentage of depolymerized polysaccharide in a commercial hexavalent liquid vaccine. Based on the method risk assessment, the critical sources of analytical variability (CMV) were identified, measured and understood. For CMVs with a high level of risk, means of control have been established and integrated in the routine use of the method. Among these CMVs, the MODR was established as proven acceptable ranges, i.e. characterized ranges for which operation within this range, while keeping other parameters constant, will produce a result meeting relevant quality criteria. After implementation of all corrective actions, residual risk evaluation was performed, and the residual risk was stated as acceptable. This optimized method, developed using the AQbD approach, was successfully submitted to the European Medicines Agency (EMA). Based on the data generated during the development study, proposed operational ranges were deemed justified and

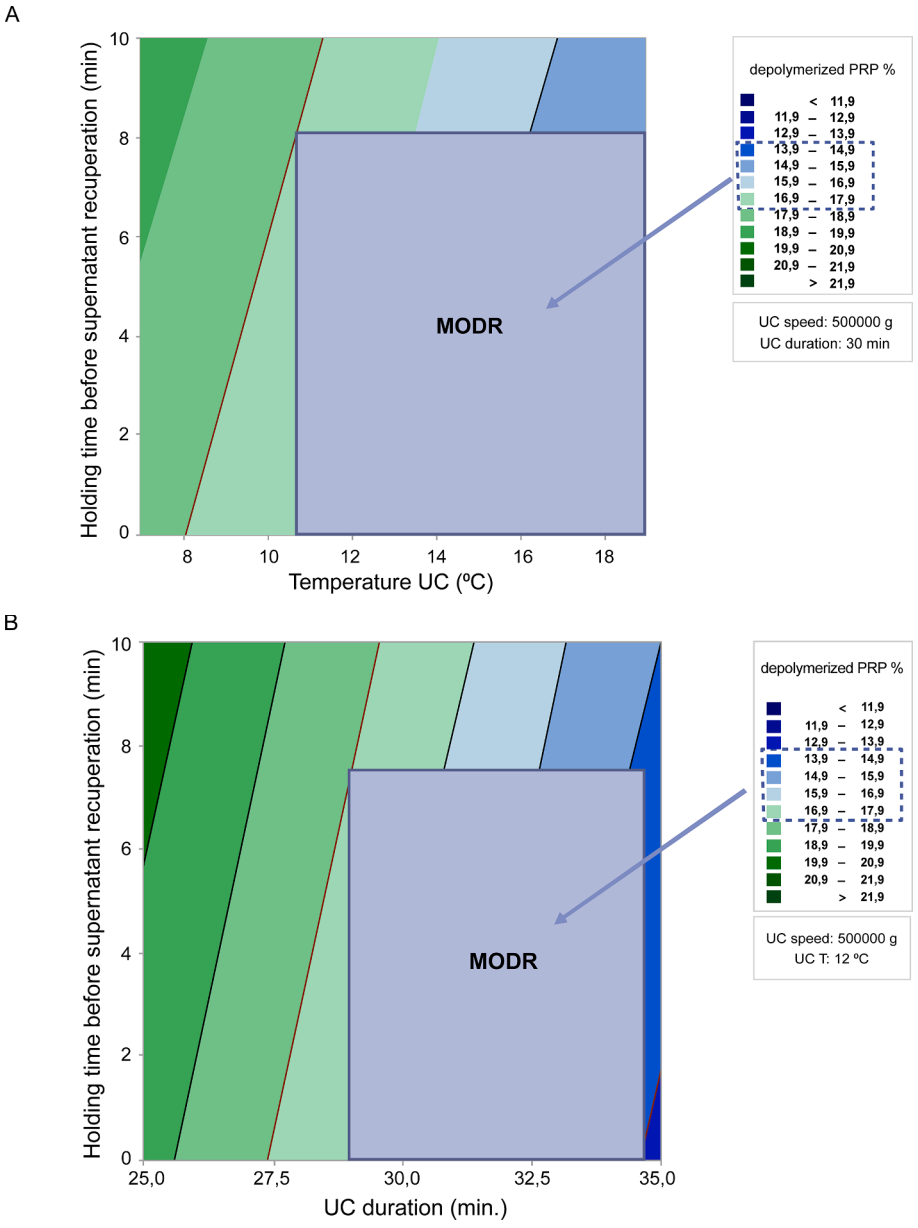

Fig. 6. . Contour map from DoE study with (A) temperature, (B) speed and (C) duration of UC, set at target values (At right, the framed values represent the acceptable result meeting the relevant quality criterion) DoE, design of experiment; PRP, polyribosyl ribitol phosphate; UC, ultra-centrifugation.

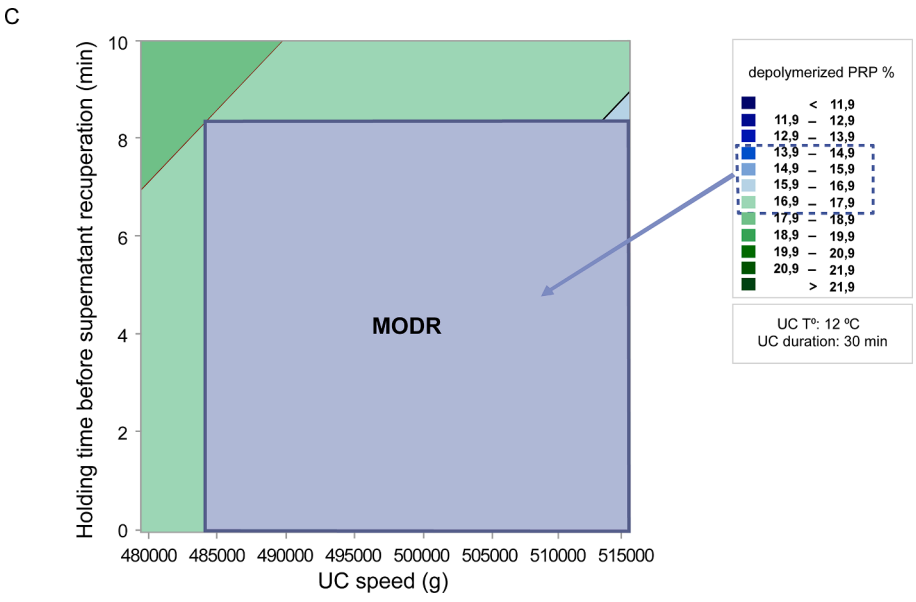

Fig. 6. (*continued*).

Table 9
Method Operable Design Region for the seven selected critical method variables.

Method parameters with potential influence on method performance	Target	Knowledge space	MODR
UC speed (g)	500,000 g	480,000–520,000 g	483,000–520,000 g
UC temperature (°C)	12 °C	7–19 °C	11–19 °C
UC duration (min)	30 min	25–35 min	29–34 min
Holding time before supernatant recuperation after the UC (min)	Extemporaneous	0–10 min	0–8 min
Helium pressure of the elution mobile phase (psi)	4 psi	2–6 psi	2–6 psi
Storage time of the elution mobile phase (day)	Day 0	0–14 days	0–14 days
Storage time of samples in the autosampler at 5 °C ± 3 °C (day)	Day 0	0–3 days	0–3 days

MODR, Method Operable Design Region; UC, ultracentrifugation

acceptable by EMA. Future analytical changes related to the proposed method, which are within the defined design region, are deemed acceptable and approvable by assessors. It would then be a great advantage for change management, providing more flexibility to implement change in analytical methods during their life cycle. In addition, AQbD applied for the development of the analytical methods is aligned with the process for analytical development that is being proposed for ICH Q14 [17] and the current United States Pharmacopeia < 1220> [8]. This concept can be applied to any method type, at any point in the method life cycle. It will result in a better understanding and fewer failures of analytical methods due to more robust methods, which will produce consistent, reliable, quality data throughout the life cycle. This will lead to fewer method transfer failures, invalid results, and method "incidents" when used in the routine environment. As the industry is now applying quality by design to process development, it is recognized that this is also the way forward to improve and standardize this innovative approach to analytical procedures.

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CRediT authorship contribution statement

Isabelle Moineau: Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **Véronique Chambon:** Investigation, Methodology, Writing – review & editing. **Céline Perret:** Investigation, Methodology, Writing – review & editing. **Luce Gouit:** Investigation, Methodology, Writing – review & editing. **Isabelle Zamora:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **Mannaig Macumi:** Formal analysis, Writing – review & editing. **Stéphanie Fertier-Prizzon:** Funding acquisition, Supervision, Writing – review & editing. **Olivier Pitiot:** Funding acquisition, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: VC, CP, LG, IZ, SP, and OP are Sanofi employees and may hold shares and/or stock options in the company. Aktehom received funding from Sanofi for study conduct and manuscript writing.

Data availability

Data will be made available on request.

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