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Review

An overview of experimental designs in HPLC method development and validation

Prafulla Kumar Sahu [a], Nageswara Rao Ramisetti [b,*], Teresa Cecchi [c,*], Suryakanta Swain [d], Chandra Sekhar Patro [a], Jagadeesh Panda [a]

[a] Department of Pharmaceutical Analysis and Quality Assurance, Raghu College of Pharmacy, Dakamarri, Bheemunipatnam Mandal, Visakhapatnam, 531162, Andhra Pradesh, India
[b] Analytical Chemistry Division, CSIR-Indian Institute of Chemical Technology (IICT), Tarnaka, Hyderabad, 500007, Telangana, India
[c] Chemistry Department, ITT MONTANI, Via Montani 7, 63900, Fermo, FM, Italy
[d] Department of Pharmaceutics, SIMS College of Pharmacy, Mangaladas Nagar, Vijayawada Road, Guntur, 522 001, Andhra Pradesh, India

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ABSTRACT

Chemometric approaches have been increasingly viewed as precious complements to high performance liquid chromatographic practices, since a large number of variables can be simultaneously controlled to achieve the desired separations. Moreover, their applications may efficiently identify and optimize the significant factors to accomplish competent results through limited experimental trials. The present manuscript discusses usefulness of various chemometric approaches in high and ultra performance liquid chromatography for (i) methods development from dissolution studies and sample preparation to detection, considering the progressive substitution of traditional detectors with tandem mass spectrometry instruments and the importance of stability indicating assays (ii) method validation through screening and optimization designs. Choice of appropriate types of experimental designs so as to either screen the most influential factors or optimize the selected factors' combination and the mathematical models in chemometry have been briefly recalled and the advantages of chemometric approaches have been emphasized. The evolution of the design of experiments to the Quality by Design paradigm for method development has been reviewed and the Six Sigma practice as a quality indicator in chromatography has been explained. Chemometric applications and various strategies in chromatographic separations have been described.

Contents

Abbreviations: 2D-HPLC, two-dimensional HPLC; AEMC, anion-exchange membrane chromatography; ANOVA, analysis of variance; BBD, Box-Behnken design; CC, chaotropic chromatography; CCD, central composite Design; CEX-HPLC, cation exchange high performance liquid chromatography; DOE, design of experiments; ELSD, evaporative light scattering detection; ESI, electrospray ionization; FD, factorial design; FFD, full factorial design; FMEA, failure mode and effects analysis; FrFD, fractional factorial design; HAC, heparin affinity chromatography; HIC, hydrophobic interaction chromatography; HILIC, hydrophilic interaction liquid chromatography; HPLC, high performance liquid chromatography; IEMC, ion-exchange membrane chromatography; ISO, international organization for standardization; LC–MS/MS, liquid chromatography tandem mass spectrometry; LSER, linear solvation energy relationship; LSS, lean Six-Sigma; mAbs, monoclonal antibodies; MEPS, micro extraction by packed sorbent; MLR, multiple linear regression; MS, mass spectrometry; NMR, nuclear magnetic resonance; OD, optimal design; OFAT, one factor at a time; OOS, out of specification; PBD, plackett-Burman design; PDA, photo diode array; PLE, pressurized liquid extraction; PLS, partial least squares; QBD, quality by design; QSRR, quantitative structure-retention relationship; RSM, response surface methodology; S/N, signal to noise Ratio; SFC, supercritical fluid chromatography; SPE, solid phase extraction; SST, system suitability test; TD, taguchi design; UPLC, ultra performance liquid chromatography; USP, United States pharmacopeia.

* Corresponding authors.
E-mail addresses: rnrao55@yahoo.com, rnrao15@gmail.com (N.R. Ramisetti), teresacecchi@tiscali.it (T. Cecchi).

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1. Introduction

Modelling a phenomenon either to describe or interpret provides an in depth understanding beyond the reach of human thinking. The two fundamental types of models generally used are theoretical and empirical. At variance with the former, empirical models do not adhere to any theoretical basis and are data-driven. Chemometrics, the science of relating measurements made on chemical systems or processes to their state via application of mathematical and statistical modeling [1] has become a well-recognized sub-discipline in contemporary analytical chemistry. The advances in high performance liquid chromatography (HPLC) analytical strategies, driven by several variables, provide large amount of data during the course of analytical measurements. Although HPLC is a versatile separation technique with wide range of applications, the process is sometimes critical due to its large number of variables, which need to be properly adjusted before every single run. Consequently, the necessity emerges for a deeper understanding of such methods. Chemometric tools with suitable statistical analysis have become popular by the way, considering multiple advantages viz. reduction in the number of experiments and lower reagent consumption and less laboratory work. Furthermore, optimization of HPLC methods are complex processes; since, several variables (mobile phase pH, buffer concentration, flow rate, column temperature, detector wave length, etc.) are to be concurrently controlled in attaining the desired separations [2]. These approaches facilitate development of mathematical models, adding valuable, scientific information in support of their ability to assess the statistical significance of the variables' influences on the desired chromatographic responses.

HPLC separation of each chemical entity from the sample mixture is based on its distinct affinity towards the adsorbent material in the column or the mobile phase, causing various constituents to travel at different velocities and separate. It was formerly called as high-pressure liquid chromatography since; it relies on high pressure pumps to allow quicker separation. Separation by HPLC predominantly depends on some intrinsic tunable parameters of mobile phase like polarity, flow rate, pH, composition and some inherent properties of sample matrix; type and nature of stationary phase; environmental factors like temperature and detector type and settings [2]. For decades, HPLC methods development was strived through trial and error approach, augmented with expertise, knowledge, and wisdom of the analyst. Use of such traditional system during optimization of the method involves changing one factor at a time while keeping others constant which can certainly achieve the desired separation, but true optimal conditions could never be promised. This customary approach of developing a HPLC method has been evidenced to be not only exaggerated in terms of time, money, and labor, but also critical to fix errors, unpredictable, and even unsuccessful [3]. A single chromatographic condition cannot address enough information concerning all the affecting factors of the HPLC process. A large number of variables need to be carefully identified, investigated and controlled thereby to overcome this difficulty. Conversely, experimental variables are interlocking and tweaking each one-at-a-time generates too much raw data, which is quite impossible to interprete for attaining a true optimal system. A chemometric experimental design can overcome this problem by planning the experiments in such a way that the data is modelled efficiently with limited number of experiments. The main objective of the design usually remains to estimate the influence of the factors and their interactions on chromatographic separation.

The significance of chemometry has been well reflected by the fact that in the recent years numerous books, book chapters, review papers and innumerable research papers have been published describing its various applications in analytical chemistry. All these works emphasize chemometric approach as an emerging tool in the field of pharmaceutical analysis. Recently, Rozet et al. discussed the use of experimental designs to define design spaces, a key component of the quality by design paradigm of analytical methods [4]. Several strategies of multivariate experimental design have been overviewed for the determination and/or removal of contaminants in environmental analysis [5]. Application of experimental designs using desirability function in context of few analytical laboratories has been reviewed for optimizing complex systems including extraction procedures [6]. Recent advances in set-up, data interpretation and applications of experimental designs have been reviewed by Dejaegher & Heyden [8]. Recent tutorial reviews by Dejaegher [7,8] and Hibbert [9] demonstrated execution of several types of design spaces for optimization and validation of analytical and separation techniques. However, a comprehensive review of applications of experimental designs in optimization and validation of liquid chromatography and allied techniques becomes necessitous. The present manuscript, describes several strategies and applications of chemometry, specially focusing on those intended mostly for optimization and validation of HPLC methods in pharmaceutical analysis.

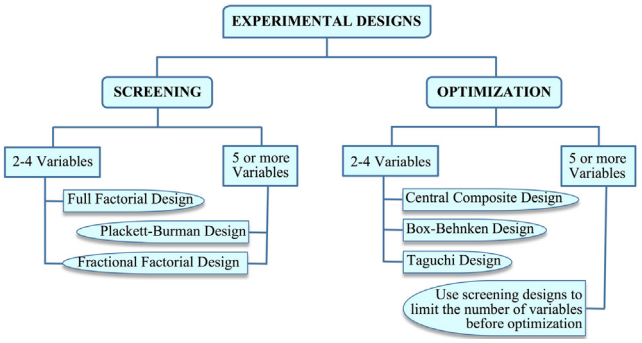


Fig. 1. Classification of experimental designs.

2. Experimental designs

Experimental designs are being often used for the optimization of several operating conditions of various processes and for improving the chromatographic separation performance, as well as attaining high extraction efficiency [10]. Ideally, a number of factors have simultaneous effect on a process. Nevertheless, identification and optimization of significant factors as a function of experimental design is most effective to achieve a competent result by fewer experimental trials. Consequently, the experimental design can be well defined as an approach to solve the problems systematically and obtain information-rich result [11]. Optimum and valid results with a minimum effort, time and resources are the primary objectives of applying the experimental design in analytical process [12]. In an experimental design, one or several predetermined factors are deliberately manoeuvred to perceive their influence on the experimental outcome.

Based on the objectives of an experiment, all the designs can be classified into two broad categories: Screening Designs, Response Surface Designs (optimization design) (Fig. 1). The kinds of experimental designs and their experimental domains have been detailed in Table 1.

2.1. Screening designs

Since a huge number of factors influence the HPLC process, some of them that do not have significant effect on it must be discarded. Screening of the most influential factors becomes the primary objective of employing experimental design in HPLC. These designs are used with a purport to identify the most important factors and their interactions from all potential factors. They are very useful to examine qualitative, quantitative and mixer-related factors simultaneously [7]. From the literature it is evidenced that Full Factorial designs (FFD), Fractional Factorial designs (FrFD) and Plackett-Burman designs (PBD) are frequently used as screening designs [8,13,14]. Such two-level designs allow screening of high number of factors with fewer experiments. Analysis of variance (ANOVA) or regression analysis can be the basis for computing effect of the studied factors on a particular response. They are frequently applied for improvement of separation techniques, formulations, products or processes of quality control and robustness [2] and ruggedness [15] testing (see Section 5). The steps to be performed in such designs are identical to that of robustness or ruggedness test with the discrepancy in the intervals within the two levels of the factors. Several applications of three or more or mixed-level screening designs also has been evidenced from the literature. Advantages and disadvantages of different screening designs are summarized in Table 2.

2.2. Response surface designs

Optimization is an additional practice of chemometric approach that endorses the optimal condition or settings of a process. Such approach usually precedes with a screening design to select the potential factors [16,17]. Response surface designs are of two types: symmetrical designs and asymmetrical designs. Three-level FFD, Central Composite design (CCD) and Box-Behnken design (BBD), Taguchi design (TD), and Doehlert designs cover a symmetrical domain with a center point to estimate experimental error. Asymmetrical designs such as D-optimal design form an asymmetrical shape when an asymmetrical experimental domain is examined. Such designs can also form a symmetrical shape in a symmetrical domain. Mixture designs are applied to study mixture variables only, i.e. to optimize the composition of mixture. ANOVA, signal-to-noise ratio and range analysis are the basis of the statistical analysis methods for response surface designs. Range analysis is used to find the effect of each factor and determine the optimal level of different factors. For a factor, the range of means is the difference of the maximum and minimum means of all levels. For a system, the factor with the largest range of means has the strongest influence on the performance. Range analysis can find the optimal value of different factors but this method cannot clearly and quantitatively determine the significance of different factors. In the ANOVA, the data are analyzed by a F-test. The F value of each factor implies the ratio of the variance for the each factor to that of the experimental error. The percentage contribution of each factor is the percentage of the sum of square deviation due to that factor in the total sum of square deviation. It reflects the factor's influence. Regression analysis enables to estimate the relationships among variables via a regression function. Linear first order and second order models are quite common. A fruitful implementation of experimental design in HPLC can be executed through four common stages; i.e.: (i) choosing the convenient design, (ii) suitable software, (iii) experimental trials, data analysis, and (iv) interpretation. The use of Optimization Designs in HPLC method development is summarized in Table 3.

3. Mathematical models in chemometry

Simply referred to as the model, is an expression defining the dependence of a response variable on the independent variables. Mathematical models can be either empirical or theoretical [3]. An empirical model provides a way to describe this factor-response relationship. It is most frequently, but not invariably, a set of polynomials of a given order. Most commonly used linear models are shown in Eqs. (1)–(3):

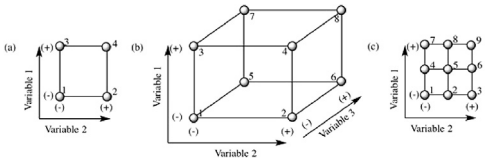
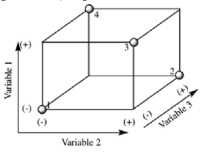
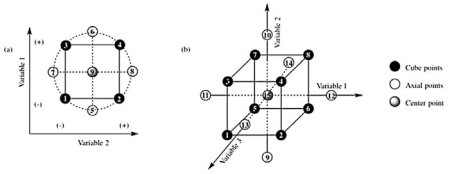
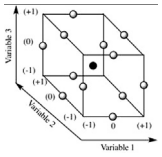
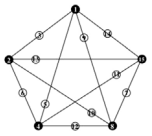
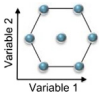
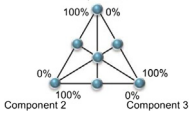
$$E(y) = \beta_o + \beta_1 X_1 + \beta_2 X_2 \quad (1)$$

$$E(y) = \beta_o + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 \quad (2)$$

$$E(y) = \beta_o + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} {X_1}^2 + \beta_{22} {X_2}^2 \quad (3)$$

Where, E(y) represents the measured response, X_1 and X_2, are the selected factors, β_0 is the intercept β_1, and β_2 are first order parameters, β_{12} is an interaction parameter, β_{11} and β_{22} are second-order parameters, respectively. The coefficients are calculated either by MLR analysis or by the method of contrasts. Eqs. (1) and (2) are linear in variables, representing a flat surface and a twisted plane in 3-D space, respectively. Eq. (3) represents a linear second-order model that describes a twisted plane with curvature, arising from the quadratic terms. The use of 3-D response surface plots allows understanding the behavior of the system by demonstrating the contribution of the independent variables. The contour plots represent the slices of response surface. Normalized plots are the graphical representation of a response plotted between normalized factor levels (N) and a response variable. These are usually

Table 1
Experimental designs and domains in liquid chromatography.

Experimental design	Level	Design space
Screening		
Full factorial design (FFD)	2 or 3-levels	 (a) Two-factor, two-level (2^2); (b) three-factor, two-level (2^3); and (c) two-factor, three-level (3^2) full factorial designs showing number of points (experimental runs).
Fractional factorial design (FrFD)	2-levels	 Fractional factorial design (2^{3-1}).
Plackett–Burman design (PBD)	2-levels	The design is analogous to a two-level FrFD. Maximum of N-1 factors with N (multiple of 4) runs can be examined in an experiment. Replicated experiments and dummy factors can be used for determining the experimental errors.
Response surface (optimization)		
Central Composite design (CCD)	3 levels	 Three level circumscribed central composite designs for (a) 2 variables and (b) 3 variables showing number of runs.
Box-Behnken design (BBD)	3 levels	 Box-Behnken design for 3 variables.
Taguchi Design (TD)	2 levels	 Linear aliased model for L16 orthogonal array (2^{15}) by Taguchi design.
Doehlert design	Multiple levels	 Doehlert design for 2 variables showing 7 runs.
D-optimal design	2-levels	 D-optimal design showing 9 runs for two factors.
Hybrid design	Multiple levels	 Hybrid design for three components with value sum of 100%.

plotted only when the levels of the factors are not equidistant, i.e., the central level is not the mean of the high and the low levels. Normalization in such cases tends to make data amenable to further specialized mathematical treatment. Normalized factor levels can be calculated using Eq. (4).

$$N = (X - L)/(H - L) \times 100 \quad (4)$$

Table 2
Pros and cons of screening designs.

Design	Pros	Cons
FFD (To optimize two to four factors)	To ascertain the effects of independent factors and their interactions.	Impossible to screen more than sixteen factors; since, increase in number of factors leads to arithmetic increment in number of trials.
FrFD (To optimize more than four factors)	More efficient than FFD since, fewer experimental trials needed for equal number of factors.	Inadequate interaction effects and may be imprudent as there is no experimental error.
PBD (To identify a small number of significant factors from a large number of experimental factors for further validation of optimization)	Large number of factors can be assessed amid very limited experimental runs.	The design is strictly suitable for identifying significant main effects and do not deem any interaction effects.

Where, X is unnormalized factor level, L is the lowest factor level and H is the highest factor level, respectively.

In FFD, with a large number of factors, it is plausible that highest-order interactions have no significant effect. In such cases, the number of experiments can be reduced in a systematic way and the resulting design is called the FrFD. FrFD is a fraction ($1/X^p$) of a complete or FFD, where p is the degree of fractionation, and the total number of experiments required for FrFD designs are given by X^{n-p}. A polynomial equation for a 2^3 factorial design can be made by expansion using the general form as shown in Eq. (5).

$$Y = B_0 + \sum_{\substack{i\\ i=1}}^{n} B_i X_i + \sum_{\substack{i\\ i=1}}^{n}\sum_{\substack{j\\ j=i+1}}^{n} B_{ij} X_i X_j + \sum_{\substack{i\\ i=1}}^{n}\sum_{\substack{j\\ j=i+1}}^{n}\sum_{\substack{k\\ k=j+2}}^{n} B_{ijk} X_i X_j X_k \quad (5)$$

Where, n = number of factors (3 in the above equation), X is +1 or −1 as per coding, the transformation formula is given as Eq. (6),

$$\textit{Transformed proportion} = \frac{\textit{Amount used} - \left(\frac{\text{maximum}+\text{minimum}}{2}\right)}{\left(\frac{\text{maximum}-\text{minium}}{2}\right)} \quad (6)$$

Y is the measured response of coefficient B_i, B_{ij} and B_{ijk} respectively and the coefficients are computed from the responses using RSM, specific for an experimental design.

The FFD or FrFD are first-degree models and their response equations at two levels have an inherent assumption of linearity. To ascertain the possibility of curvature, second-degree models are required. CCDs are effective second-degree designs, which combine the advantages of the FFD (or FrFD) and the star design. These designs, also known as Box-Wilson designs, provide maximum information with minimum experimentation. This design consists of a central core of a two level factorial design (2^n), one central point and 2n outer points (in pairs along the co-ordinate axes at $\pm\alpha_1$, $\pm\alpha_2$,... $\pm\alpha_x$, respectively, where α is the distance of axial points from the center) corresponding to a star design [3]. The calculation of effects and interactions, coefficients of the equations, and the statistical significance of coefficients, so generated can be carried out using various methods. The method of contrasts and Yates algorithm can be used for calculating the effects and interactions. The coefficients can be calculated either by regression analysis or by the method suggested by Davies. The significance of coefficients can be computed by applying ANOVA based on Yates method or by Student's *t*-test. The polynomial equation for a CCD can be made using the expansion of the general form given in Eq. (7).

$$Y = B_0 + \sum_{\substack{i\\ i=1}}^{n} B_i X_i + \sum_{\substack{i\\ i=1}}^{n}\sum_{\substack{j\\ j=i+1}}^{n} B_{ij} X_i X_j + \sum_{\substack{i\\ i=1}}^{n}\sum_{\substack{j\\ j=i}}^{n} B_{ii} X_i^2 \quad (7)$$

The coefficients B_i, B_{ij} are computed from the observed response Y. The value of X would be 0 or ±1 or ±α as per the required CCD.

Mathematical optimization methods are used to seek an optimum formulation by solution of the equation (objective function) for either a maximum or a minimum in presence of equality and/or inequality constraints [3]. Objective function may be expressed in Eq. (8) and the inequality and equality constraints are expressed in Eqs. (9) and (10).

$$Y = f(X_1 X_2) \quad (8)$$

$$G(X) = f_1(X_1, X_2) \geq 0, \text{ i.e., inequalityconstraint} \quad (9)$$

$$H(X) = f_2(X_1, X_2) = 0, \text{ i.e., equalityconstraint} \quad (10)$$

If the objective function is expressed as a function of a single variable, i.e., Y = f(X) classic optimization techniques resulting from application of calculus to the basic problem of finding the maximum or minimum of a function can be applied. Its first derivative can be taken and by setting it equal to zero can be solved for X to obtain the maximum or minimum. When the relationship for the response Y (objective function) is given as a function of two or more independent variables as in Eq. (11) for X_1 and X_2 the problem is slightly more involved. Mathematically, appropriate manipulations with partial derivatives of the function can locate the necessary pair of X values for the optimum.

$$Y = F(X_1, X_2) \quad (11)$$

In the presence of multiple response processes, the desirability function approach is needed for their optimization. The method searches operating conditions that provide the "most desirable" response values. For each response, a desirability function transforms the values of a response into [0,1] where 0 stands for a completely undesirable value of the response and 1 for a completely desirable or ideal response value. The individual desirabilities are then combined using the geometric mean, which gives the overall desirability [6].

4. Application of chemometric designs

Application of chemometry allows investigators to understand multiple method parameters and variables that tend to impact critical responses, whilst unraveling the occurrence of

Table 3
Optimization designs used in HPLC methods development.

Design	Sample/API	Matrix	Software used	Factors Optimized	Ref.
Taguchi	*Candida tropicalis* PHB5	4-chlorophenol	Qualitek-4 software (NutekInc., MI, USA)	Eleven factors ($MgSO_4$, phosphate ion, NaCl, yeast extract, $CaCl_2$, temperature, pH, inoculum size, incubation time, agitation, trace element solution)	[18]
Taguchi	Sumatriptan succinate	Niosomes	DOE Pack® 2000 software	Four factors (ACN in mobile phase, pH buffer, oven temperature, flow rate)	[19]
Taguchi	Bioactive compounds	Soil bacteria	Information not available	Four factors (nutrient concentration, temperature, salt concentration and pH)	[20]
BBD	Isomers β-caryophyllene and α-humulene	Phytotherapies and cosmetics	Version 10.1 Statistica® (Stat-Ease Inc., Minneapolis, MN, USA) and version 5.0 GraphPad Prism® software (GraphPadSoftware,LaJolla,CA, USA),	Four factors (flow rate, gradient slope time, proportion of ACN at the end and beginning of the gradient)	[21]
BBD	Polycyclic aromatic hydrocarbons (PAH)	Rainfall water	Statgraphics Plus 4.1" (ManugisticsInc, Rockville, MA, USA)	Three factors (desorption time, temperature and PAH concentrations)	[22]
BBD	Phthalate esters	Cosmetic and environmental water samples	StatGraphics Plus Package, version 5.1	Four factors (extraction solvent volume, salt effect, extraction time and centrifugation time)	[23]
BBD	Aliphatic C_1–C_{10} aldehydes	2,4-dinitrophenylhydrazine (DNPH)	Startgraphics Plus Version 3 for Windows (Manugistics, Rockville, USA)	Four factors (time, temperature, excess of reagent and stirring onto the reaction yields)	[24]
BBD	Amoxicillin trihydrate	Bulk and pharmaceutical formulations.	Design-Expert 8 software	Three factors (composition of mobile phase, flow rate and pH)	[25]
BBD	*Rhizoma Salviae Miltiorrhizae* (Danshen)	Danshen and its preparations	SAS 9.0 (SAS Institute Inc., Cary, USA)	Two factors (Flow rate and solvent gradient)	[26]
BBD	2-Methyl-6-nitroaniline and 2-methyl-4-nitroaniline	Preparations	Microsoft Excel Program (Microsoft Corporation) and Design Expert 7.0 Software (Stat-Ease, Inc. Minneapolis, MN)	Three factors (column temperature, flow rate, and the percentage of eluent)	[27]
BBD	Metaxalone	Bulk/Tablets	Design-Expert software, Trial Version 9, Stat-Ease Inc., Minneapolis, MN, USA)	Three factors (column temperature, buffer pH, and buffer concentration)	[28]
BBD	Ezetimibe	Supersaturable Self-Nanoemulsifying Formulation	Minitab	Five factors (% organic phase, buffer pH, flow rate, injection volume, column temperature)	[29]

Table 3 (*Continued*)

Design	Sample/API	Matrix	Software used	Factors Optimized	Ref.
BBD	Metaxalone	Bulk/Tablets	Design-Expert software, Trial Version 9, Stat-Ease Inc., Minneapolis, MN, USA)	Three factors (buffer pH, % organic phase and flow rate)	[30]
CCD	Oleuropein	Olive's processing wastewater (OPW) and olive leaves extracts	MINITAB software, version 15, Minitab Inc.Pensylvania.	Five different parameters influencing the extraction efficiency of the method	[31]
CCD	Chlordiazepoxide	Four different matrices of real samples (water, urine, plasma, and chlordiazepoxide tablet)	STATISTICA statistical package software version 7.0 (Stat Soft, Tulsa, USA)	Six factors (volume of chloroform, methanol, extraction time, time of centrifugation, pH, % NaCl)	[32]
CCD	Domperidone and pantoprazole	Commercial pharmaceutical preparations	Design-Expert® trial version 7.0.0. (Stat-Ease Inc., Minneapolis)	Three factors (mobile phase composition, buffer molarity and flow rate)	[33]
CCD	10 Diterpenoid compounds	Chinese medicine (*salvia miltiorrhiza*)	Version 6.0 (StatSoft, Inc., 2001)	Three parameters (gradient, flow rate, and column temperature)	[34]
CCD	Sudan dyes	–	Information not available	Three factors (mobile phase composition, flow rate and column temperature)	[35]
CCD	Amlodipine and atorvastatin	Pharmaceutical formulations	Design-Expert trial version 7.0.0. (Stat-Ease Inc., Minneapolis)	Four factors (mobile phase; buffer pH; buffer concentration; and flow rate)	[36]
CCD	Azithromycin, secnidazole, and fluconazole	Formulation (combi-kit)	Design-Expert trial version 9.0.0. (Stat-Ease Inc., Minneapolis)	Two factors (% organic phase (MeOH) and flow rate)	[37]
CCD	Moxifloxacin HCl and ketorolac tromethamine	Eye drops	Design-Expert® version 8.0.4.1.	Three factors (methanol content in the mobile phase composition, buffer pH and flow rate)	[38]
CCD	Furosemide	Bulk drug	Design-Expert 8 (trial version) software	Two factors (organic modifier percentage, and flow rate)	[39]

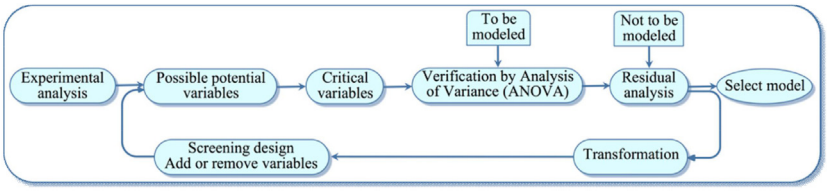


Fig. 2. MLR modeling in experimental designs.

(any) interactions and diminishing complexities. For the successful implementation of chemometric study, the knowledge of response variables, critical method variables, their ranges, and suitable mathematical model(s) is mandatory. Response surface methodology (RSM) based various chemometric designs are effective in systematic development of analytical methods comprising significant variable-response relationship(s) [40]. The experimental designs assist in mapping the responses on the basis of the studied objective(s) and exploring the critical responses at high (coded as +1), medium (coded as 0), or low (coded as −1) levels of the variables. It tends to reveal the mechanistic understanding of the variables-responses relationship, and their associated interactions via various pictorial/graphical tools. 3D and 2D-plots like response surface plots, contour plots, perturbation plots, linear correlation plots, outlier plot and Box-Cox plot are very useful in this regard [41]. Once, data acquired by the chosen design have been collected, the results can be analyzed using statistical methods like Multiple Linear Regression (MLR) analysis so that objective conclusions can be drawn [42]. The flow layout of a typical chemometric-based regression model employed for method development and data analysis is illustrated in Fig. 2.

4.1. HPLC method development

A pictographic position of HPLC staring at numerous influential factors and the assessment of outcome by experimental design is shown in Fig. 3. The Fish-Bone diagram depicts that several factors (i.e., controlling variables and noisy variables) can attribute to bring variability in method desirability criteria (response variables). The purpose of using multivariate approach via design of experiment (DOE) tools is to optimize the response variables of a sample (mixture) by logical adjustment of all controlling variables simultaneously. In contrast, a typical univariate approach will miscarry to convince the desirability and is unfeasible. The selection of experimental design for HPLC may depends on the goals of the study, investigators' interest, feasibility of experiment, cost-effectiveness, time consumption and so on. For example, to find the most potent factors in a particular experiment, two level factorial designs (FDs) can be the choice whereas to optimize the previously found influential factors within a predetermined range, more complex designs like CCD or BBD are applicable [43].

4.1.1. Dissolution studies

Dissolution test is one of the pharmacopoeial prerequisite for evaluation of solid dosage forms. The optimal dissolution medium becomes more significant when the tablets under study contain more than one active pharmaceutical ingredient of discrete chemical structures and solubility. The best dissolution criteria for a particular tablet formulation is desired to provide maximum drug release within specific time period in order to comply pharmacopoeias and regulatory specifications. It becomes more challenging task when the recovery study for the samples withdrawn form the dissolution medium is to be analyzed by HPLC. Certainly, chemometrics (screening or optimization designs) becomes more useful in setting the parameters of dissolution and HPLC process to achieve satisfactory results.

Gumieniczek and coauthors proposed the first ever HPLC method for an *in vitro* dissolution study of tablets containing binary mixture of amlodipine and perindopril. Robustness of the procedure was tested with the aid of PBD to estimate the method suitability. In addition to HPLC variables (percentage acetonitrile, pH, flow rate of the mobile phase, column temperature) the factors related to dissolution (pH and percentage content of cetylpyridine chloride in the dissolution medium and rotation speed of the paddle) were investigated for percentage recovery of the both drugs [44].

4.1.2. Sample preparation and matrix effects

Pretreatment of biological matrices is an important prerequisite in chromatographic determination of analytes in order to remove proteins and other impurities therein. Several sample preparation procedures such as protein precipitation, solid-phase extraction (SPE) or liquid–liquid extraction include multistep sample pretreatments and are often dictated by expensive, time consuming and unsuitable for emergency medicine. Hence, an increasing demand for use of microsorption techniques (solid phase or liquid phase microextraction) was found in the recent literature. However, a large number of factors may affect the extraction efficiency (sample volume, temperature, extraction time, stirring speed, % organic modifier, ionic strength and pH) or liquid desorption efficiency (type of solvent, temperature, time, number of successive steps and mode [magnetic stirring or ultrasounds]) of sorption techniques. The extraction conditions must be optimized with the aid of response surface methodology based chemometry, since the particular factors are not considered during the analytical process.

Bazhdanzadeh et al. recently reported a new stir bar sorptive extraction and HPLC-UV method for simultaneous determination of chlorpromazine and trifluoperazine in human serum. Factors (sample volume, extraction time, temperature and pH) affecting the best extraction and desorption efficiency were investigated and optimized using a screening 2^5half FrFD followed by a BBD experimental design [45]. Dawes and coworkers illustrated the benefits of performing design of experiments (DOE) in clinical assay of human plasma by LC–MS/MS. The sample extraction procedure (liquid–liquid extraction) was optimized for three factors, extraction buffer pH (two pHs), volume ratio of organic solvent to plasma (two ratios), and extraction shake time (three times), using $2^2 \times 3$ FFD to yield greater recovery of analytes from the extraction, the response ratio of each analyte over the internal standard, and to estimate matrix effects. Conventionally preferable longer extraction time (shake time) may cause decreased analyte response due to increased extraction of interfering matrix components (matrix effects). Owing to the fact that the influence of extraction time is analyte dependent, the shake time must be investigated during the method development [46]. Ebrahimzadeh et al. demonstrated an Ion-pair based hollow fiber liquid phase microextraction coupled with HPLC-UV for the preconcentration and quantification of methimazole in biological samples and animal feed. The significant factors (source phase pH, extraction time, amount of $NaClO_4$

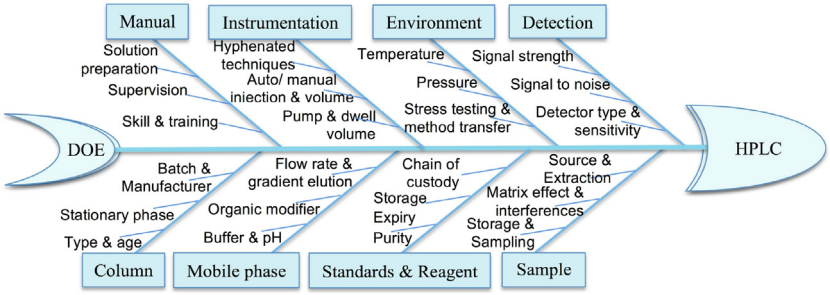


Fig. 3. Experimental design in HPLC method development and validation.

and CTAB concentration) affecting the extraction efficiency were screened by PBD prior to optimization by CCD [47]. Iriarte et al. employed FrFD and CCD to optimize a SPE-HPLC-UV-fluorescence method for extraction and separation of valsartan and its metabolite valeryl-4- hydroxy-valsartan from human plasma samples. From the FrFD investigation, the significant variables for SPE (buffer solution concentration, drying time, and elution liquid volume) and HPLC process (flow rate of the mobile phase, pH value, initial ACN%, and gradient steepness) were screened and optimized by CCD [48]. A rapid, convenient and appropriate extraction and analysis method (microwave-assisted extraction-HPLC-PDA) was published for determination of oleanolic acid and ursolic acid in the fruits of *Chaenomeles sinensis*. CCD was employed to optimize the extraction process variables (microwave power, temperature, solvent to material ratio and extraction time) [49].

In a recent study, Yazdi et al. applied HPLC–UV assisted surfactant-enhanced hollow fiber liquid phase microextraction for the extraction and analysis of melamine in soil samples. Different factors were screened (FrFD) and optimized (CCD) to achieve best extraction yield. Surfactant concentration, sample pH, extraction time and stirring rate were found as significant factors to influence the extraction efficiency. The optimized technique is advantageous to provide easier and faster simultaneous extraction and clean up for determination of melamine in complex matrix like soil [50]. A BBD aided optimization of high-pressure machine decocting process for HPLC fingerprints of Dachengqi Tang was discussed recently (Xie et al.). Influence and interactions of the process variables (soaking time, the volume of solvent and pressure) were investigated for six responses (the contents of hesperidin, aloe-emodin, honokiol, magnolol, and emodin) and the model based synthetic scores. Such method can be useful for extraction and analysis of thermolytic compounds [51].

Latest developments in sample preparation by microextraction techniques have been directed toward miniaturization and automation. If suitably optimized, microextraction by packed sorbent (MEPS) provides lower extraction time and cost, minimal matrix effect and maximum efficiency. Magiera et al. employed PBD to fine-tune the important factors (type of eluting solvent, solvent pH, elution volume, sample pH, sample volume, duration of sorbent drying and washing solvent) that could impair the MEPS efficiency for extraction and quantitative analysis of nonsteroidal anti-inflammatory drugs in urine by RP-UHPLC [52].

A multi-criterion CCD based experimental design with desirability function was used for SPE extraction and analysis of quinolones (ciprofloxacin, danofloxacin, enrofloxacin, difloxacin and flumequine) in turkey muscle by LC–UV, LC–MS and LC–MS/MS detection. Amount of organic modifier (%acetonitrile) and acid (%o-phosphoric acid) each at three levels were studied for global desirability in extraction of quinolones (Clemente et al.) [53].

A reliable pressurized liquid extraction (PLE) procedure was developed to quantify ultra-trace levels of 10 alkylphenols and 12 pesticides in real environmental samples by LC–MS/MS. A two level FFD was employed to investigate the influence of different factors (temperature, pressure, duration and number of cycles) for best extraction recoveries of the analytes from sludge, suspended materials, atmospheric fallouts and roof deposit (Baugros et al.) [54]. Rodriguez and co-authors developed a PLE based LC with fluorescence detection method for the determination of residues of fluoroquinolones in infant food products. The suitability for best PLE extraction was investigated by screening of four significant process parameters by FrFD design (% ACN/o-phosphoric acid, temperature, pressure and number of extraction cycles) and subsequent optimization by a face centered CCD (% ACN/o-phosphoric acid, extraction temperature and pressure) for best recovery [55].

Soderblom et al. described a LC-ESI–MS/MS based label-free quantitative phosphoproteomics workflow utilizing a TiO_2 enrichment protocol that enabled direct analysis of small amounts biological matrix [wild-type zebrafish (*Danio rerio*) embryo lysate]. Prior to LC–MS analysis, TiO_2 enrichment protocol was optimized for high recovery of phosphopeptides with reduced percent of nonphosphorylated peptide interference. The optimization was achieved by assessing a twenty-point condition matrix of binding modifier (MassPrep Enhancer) concentrations and peptide-to-resin capacity ratios [56].

4.1.3. LC–MS/MS determinations

HPLC optimization and separation of analytes play a significant role with the progressive substitution of traditional detectors with multiple MS instruments. Since LC–MS involves highly complex instrumentation, separation and detection become more critical. In addition to different LC factors (see Section 1), several MS parameters including cone voltage, collision energy, ionization time, temperature, quadrupole resolution etc. can influence the analytes' separation and detection. Necessarily, such critical variables must be fine-tuned in order to achieve the best ion abundance, resolution and detection. Most of the time, an optimized sample preparation also is one of the important prerequisite for LC–MS analysis of compounds in biological matrices (see Section 4.1.4).

Complex biological samples may influence (suppression/enhancement) the MS ionization of the analytes of interest due to matrix effect. Electrospray ionization (ESI) is the most frequently used technique in the quantitative applications of LC–MS in metabolomics and interactomics, among others. Several instrumental parameters are known to affect analyte ESI–MS response (analyte signal intensity and quality or ion abundance) and require DOE-based optimization. Raji and Schug employed FFD as a DOE tool to investigate the effects of four different ESI–MS parameters (spray voltage, tube lens voltage, capillary temperature and capillary voltage) on the analyte signal intensity of three tripeptides (Gly-Phe-Gly; Gly-Glu-Gly; and Gly-Arg-Gly) with the aid of Yates' algorithm [57].

Székely et al. demonstrated a one-stage DOE supported LC–MS/MS method for trace analysis of 4- dimethylaminopyridine

(genotoxic impurity) in glucocorticoids. A face centered CCD was employed to simultaneous investigation of 3 LC factors (flow, gradient and injection volume) and 2 MS factors (cone voltage, collision energy). The method was further accomplished with a robustness test for variables such as column temperature and quadrupole resolution using a FFD [58]. Székely and coauthors discussed a DOE assisted optimization of a LC–MS/MS method development for the trace analysis of the potentially genotoxic 1,3- diisopropylurea impurity in mometasone furoate glucocorticosteroid. The LC and MS factors studied simultaneously include: flow, gradient, injection volume, cone voltage, and collision energy. The robustness of the optimized method was tested using FrFD [59]. Recently, Hecht' et al. demonstrated the utility of a new class of DOE, such as the definitive screening design for optimization of mass spectrometry parameters increased glycan abundances during FANGS-INLIGHT purification process. They sought to optimize 12 MS parameters including S lens, ionization time, temperature, voltage, underfill ratio, window, ionization time (MS2), stepped normalized collision energy (midpoint), stepped normalized collision energy (step size), automatic gain control (MS1), automatic gain control (MS2), resolution (MS1) to achieve 3 fold increased ion abundances in contrast to the most reliable SPE technique [60].

4.1.4. LC–MS data processing in metabolomics

Worldwide, metabolomics, the study of metabolites has increasingly viewed as a precious complement to routine investigative work, adding valuable, scientific information in analysis of biological samples. Unanimously, hyphenated techniques (e.g., LC–MS and LC-NMR) have been proved as valuable tools in metabolomics with advantages of efficiency, high sensitivity and covering a wide range of metabolites. Although several softwares (MetAlign, MetaboAnalyst, MZmine, and XCMS) have been developed and used for LC–MS data processing (noise filtering, peak detection, identification, and alignment), parameter settings became always an inevitable problem. Certainly, from the recent literatures, it has been well proven that application of DOE in optimal XCMS parameter setting can bring huge improvement in the reliability index, data quality, meaningful unknown peaks identification, noise control and time saving in contrast to standard parameter setting.

LC–MS analysis of human urine supported by three-step DOE (Step 1: PBD; Step 2: CCD; Step 3: CCD) protocol was developed for processing of metabolomics data and compared with the default setting. A total of 17 parameters (including 6 qualitative and 11 quantitative parameters) in the XCMS setting were selected for three-step optimization to maximize the reliability index. The developed optimization protocol was proved to be an effective and faster approach for LC–MS data processing to improve LC–MS metabolomics data quality [Zheng et al.] [61]. A strategy for optimizing UPLC–MS metabolomics data processing parameters by a sequential DOE approach was proposed by Eliasson et al.. The concept was based on a dilution series from a pooled sample and a measurement of correlation between diluted concentrations and integrated peak areas and focused mainly on peak detection. Two different DOE approaches [multiple linear regression (MLR) and partial least-squares (PLS)] have been investigated for best reliability index [62].

4.1.5. Stability-Indicating assays

Chromatography is a commonly employed advanced technique for purification of compounds from complex mixtures. Chromatographic purity has proved as valuable quality index for any reference standard. A reliable HPLC method is desired to separate the analytes of interest from impurities such as related substances, natural contaminants, process or stress related impurities of similar chemical properties that may form during storage. Unlike simple LC method development, stability-indicating assay methods are more complex due to formation of several unknown degradants during the study under different stress conditions. Several factors must be fine-tuned to achieve complete peak separation between the target compound and impurities and require multivariate statistical optimization.

A BBD based RSM was used to optimize several chromatographic variables (contents of ACN, perchloric acid, triethylamine, and column temperature) in the development of a stability-indicating HPLC method for the purity determination of yunaconitine reference standard. The optimal condition was established by Derringer desirability function to meet best resolution with an acceptable analytical time [63]. Carini et al. employed experimental designs for the optimization and validation of a stability-indicating HPLC method for quantification of Achyrobichalcone. BBD design was employed for optimization of the chromatographic condition (column temperature, flow rate and acetonitrile content in the mobile phase) for desired responses (resolution between the impurities, resolution between the main analyte and adjacent impurity and retention factor of main analyte). Robustness of the method was determined by PBD [64].

Very recently, a DOE based quality by design (QBD) approach has been used for establishing the normal operating range of a ternary gradient robust HPLC method for simultaneous determination of related substances of Abacavir, Lamivudine, and Dolutegravir in combined drug product. Based on the identified critical attributes and variables from the established OFAT-HPLC method, an initial 2^3 FFD was failed and augmented to CCD optimization of 3 significant factors (buffer pH, temperature and mobile phase composition B).Since, the two of the critical responses (Lamivudine carboxylic acid retention time and resolution between Abacavir impurity B and unknown impurity) require two distinct pHs, the design spaces became impractical due to high probability of failure. Hence, process capability analysis (Cpk values) for various responses was performed by Monte Carle simulation (Minitab, USA) to demonstrate the robustness of the method (Tol et al.) [65].

A QBD based simple and sensitive stability-indicating RP-HPLC method has been reported for the estimation of olmesartan medoxomil. Screening of various process parameters (mobile phase ratio, flow rate, injection volume, column oven temperature, flow type, column type, and column dimension) entails investigation of critical method variables affecting critical analytical attributes (theoretical plates and peak tailing) by a two level TD. A MLR analysis of the effects of critical method variables and their interactions on the studied responses was performed for subsequent optimization of the chromatographic condition (Beg et al.) [66].

4.1.6. Ultra performance liquid chromatography (UPLC)

UPLC is a modern analytical technology that surpasses the victory of HPLC with advantages of use of stationary phase with smaller particle size (1.7 μm) operated at elevated pressure to achieve unchallenged rapid separation, resolution, and sensitivity. With the increasing success of UHPLC instrumentation, utility of various chemometric tools can further enrich the best separation results. The recent literature survey reveals that tremendous efforts has been paid to optimize UPLC analysis of complex mixtures of compounds by multivariate approach.

Li et al. reported a first in class UPLC method for quantitative analysis of 10 diterpenoids (tanshinone I, tanshinone IIA, cryptotanshinone, dihydrotanshinone I, 1,2-dihydrotanshinquinone, methylenetanshinquinone, miltirone, 5,6-dehydrosugiol, sugiol, and przewalskin) in *Salvia miltiorrhiza,* a traditional Chinese medicine. A CCD based DOE tool was employed to investigate the critical variables (Gradient of organic modifier (time), flow rate and column temperature) of the method that could influence the UPLC resolution [34].

4.1.7. Miscellaneous chromatographic applications

Rodriguez-Nogales and co-workers employed a CCD (2^4 + star) based multivariate analysis technique to optimize gradient elution of RP-HPLC method for the characterization of maize proteins using monolithic columns. Significant factors such as ΔACN_1(%); Δt_1 (min); ΔACN_2 (%), and Δt_2 (min) were optimized for the HPLC using a monolithic column to enable the drastic reduction in separation time (to 8.3 min) usually employed to separate maize proteins with conventional columns (60–80 min) [67].

Valliappan et al. developed a DOE assisted HPLC method for prediction of chiral separation of ketoprofen. A 2^3 complete FD was employed as a prospective tool to optimize and predict resolution of L-leucinamide derivatives of (±)-ketoprofen and their retention times. Good separation of the resultant diastereomers of chiral ketoprofen (formed by derivatization using L-leucinamide and ethylchloroformate at ambient temperature) was accomplished by identifying the optimal limits of the critical process parameters (% organic modifier, buffer concentration and flow rate) by the design space [68].

Two successive experimental designs (design I: FD; design II: CCD) were employed to optimize triple-potential waveform (E1: determination potential; E2: cleaning potential and E3: activation potential) for HPLC with pulsed amperometric detection of streptomycin and dihydrostreptomycin in veterinary drugs. The method was complemented by a robustness test to examine the effects of seven factors using a PBD (Martínez-Mejía and Susanne Rath) [69].

Upon reexamining, Snyder and Dolan explored that the changes in temperature or mobile phase composition (% organic modifier) were cross-purposed to be responsible for changes in HPLC selectivity inmost of the studies. Nevertheless, a change of column or mobile phase pH can more significantly influence the retention behavior of an analyte. It was recommended that reversed-phase retention (δ log k) as a function of sample and other parameters as a function of conditions (temperature, %B, relative concentrations of acetonitrile and methanol in the mobile phase and buffer pH) must be considered in optimizing the selectivity [70].

5. HPLC method validation

In recent years, scholarly literatures on use of chemometrics, up to this point, encompassed experimental designs not only for method optimization, but also for method validation. Several validation parameters especially robustness, ruggedness, system suitability test (SST), were evidenced to furnish more valid results when assessed using chemometry. When a new method is optimized it is important to establish how robust or rugged it is. However, there is huge misperception in most literatures concerning the use of the terms robust/robustness and rugged/ruggedness for the description of analytical methods. Most publications use them as if they were synonymous. The critical review by Burns et al. distinguished the ambiguity of the terms robust/robustness and rugged/ruggedness and recommended the following definitions [71].

Robustness: "*Robustness of an analytical method is the property that indicates insensitivity against changes of known operational parameters on the results of the method and hence its suitability for its defined purpose.*"

Ruggedness: "*Ruggedness of an analytical method is the property that indicates insensitivity against inadvertent changes of known operational variables and in addition any variations (not discovered in intra-laboratory experiments), which may be revealed by inter-laboratory studies.*"

The **Relative Robustness** value determined in an intra-laboratory experiment can express the method's robustness, while **Relative Ruggedness** from inter-laboratory studies defines method's ruggedness. The mathematical models are followed for determination of robustness (Eq. (12)), relative robustness (Eq. (13)), ruggedness (Eq. (14)) and relative ruggedness (Eq. (15)) of an analytical method. Higher the value of relative robustness or relative ruggedness better will be the method's robustness or ruggedness.

$$rob\left(\frac{A}{B},\ldots,N;f_1,\ldots,f_m\right)=\frac{1}{\sum_{i=B}^{N}|S_{Ai}|x_i+\sum_{j=1}^{m}|I_{Aj}|x_j} \tag{12}$$

$$rob_{rel}\left(\frac{A}{B},\ldots,N;f_1,\ldots,f_m\right)=\frac{S_{AA}x_A}{S_{AA}x_A+\sum_{i=B}^{N}|S_{Ai}|x_i+\sum_{j=1}^{m}|I_{Aj}|x_j} \tag{13}$$

$$rug\left(\frac{A}{B},\ldots,N;f_1,\ldots,f_m;u_{1,\ldots,u_p}\right)=\frac{1}{\sum_{i=B}^{N}|S_{Ai}|x_i+\sum_{j=1}^{m}|I_{Aj}|x_j+\sum_{k=1}^{p}|u_k|} \tag{14}$$

$$rug_{rel}\left(\frac{A}{B},\ldots,N;f_1,\ldots,f_m;u_{1,\ldots,u_p}\right)=\frac{S_{AA}x_A}{S_{AA}x_A+\sum_{i=B}^{N}|S_{Ai}|x_i+\sum_{j=1}^{m}|I_{Aj}|x_j+\sum_{k=1}^{p}|u_k|} \tag{15}$$

(A, analyte; $i=B, \ldots, N$, accompanying species; $f_j(j=1, \ldots, m)$, influencing factors; $|S_{Ai}|$ cross sensitivities and x_i, their actual amounts; $|I_{Aj}|$, specific influencing strengths and x_j, their actual values; $S_{AA}x_A$, ideal signal; $u_k(k=1, \ldots, p)$, unknown interferents and $|u_k|$, their effects)

5.1. Robustness

Robustness can be assessed by statistical experimental design. The chromatographic factors studied in a SST such as resolution, efficiency, capacity factor, peak asymmetry factors, etc., can also be viewed as responses in a robustness test. It is worth noticing that, it is possible to define system suitability limits based on the evaluation of the robustness because it explores the most extreme variations in the factors that may occur. Since it is rare to get a globally satisfactory solution, this procedure would avoid ambiguous situations. Different steps in robustness testing of chromatographic methods are presented in Fig. 4. Based on the objective, two strategies can be adapted for robustness studies. If the investigation only meant to verify that the already validated method is robust, screening designs such as PBD or supersaturated design is employed. In case of an experimental model need to determine the robust domain (tolerable variations) via response surfaces, preferably optimization designs such as CCD or BBD can be considered. A recent review recommends the two-level FFD for robustness evaluations of analytical methods. However, the most commonly applied screening designs in robustness tests are FrFD or PBD designs due to operational convenience [72]. Youden test (FrFD) for robustness evaluation of chromatographic analysis of compounds in various matrices has extensively reviewed [73].

5.1.1. Literature review for robustness test of LC methods

Several experimental designs containing different numbers of experiments (N) [Plackett–Burman ($N=8$ or 12) and supersaturated designs ($N=6$)] were compared for robustness testing [74]. A stepwise guidance in setting-up and interpreting a robustness test was reported combined with derivation of system suitability

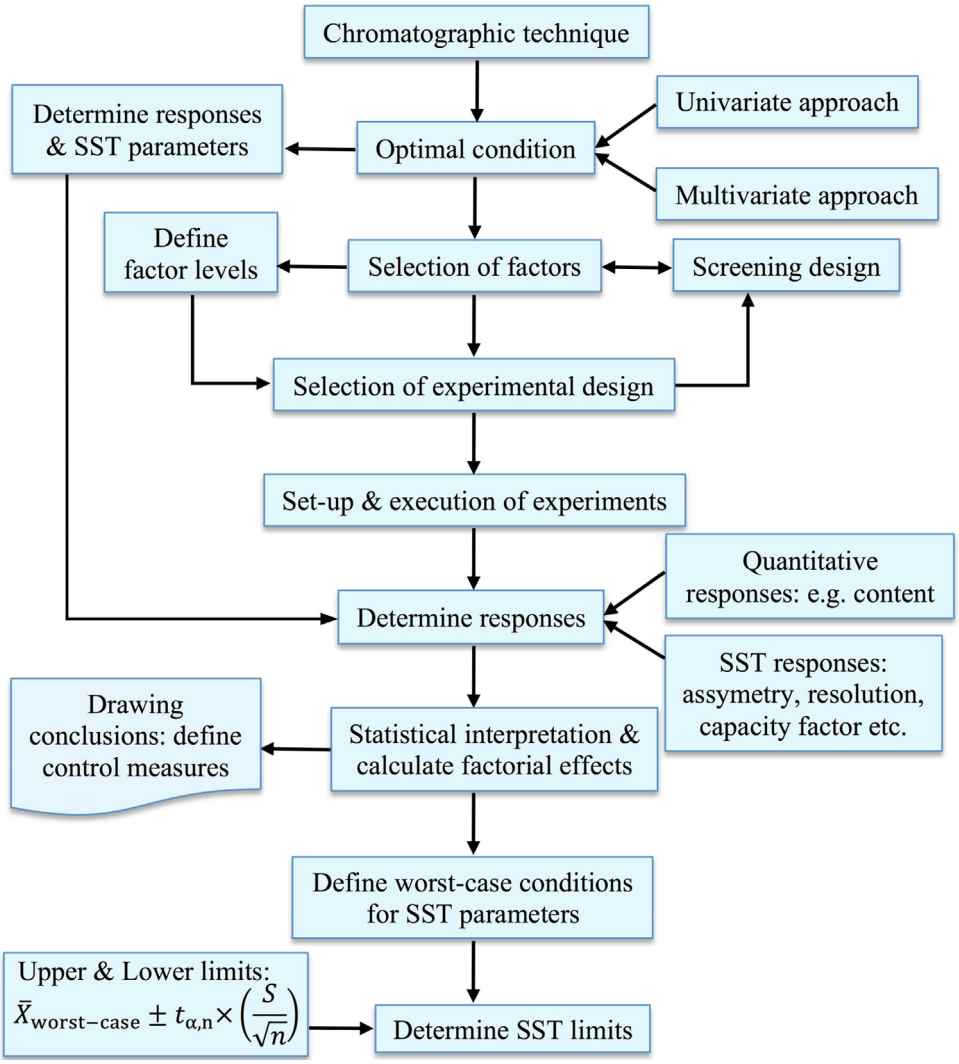


Fig. 4. Robustness testing and SST determination.

limits from robustness test results based on worst-case condition [2]. Robustness of high throughput HPLC based plasma glycan analysis was tested [75]. The accuracy of simulated robustness test of a LC method was evaluated using modeling software (DryLab) and state-of-the-art stationary phases (short narrow bore columns of 50 × 2.1 mm packed with sub–2 μm particles) for the separation of amlodipine and related impurities [76]. A case study was reported for *in silico* robustness of purity testing of pramipexole is performed adapting a compendial method based on Quality-by-Design principles [77]. The central composite design was adapted for the robustness study of a HPLC method for quantitative analysis of zileuton [78].

Sousa et al. developed an HPLC-ELSD method to determine six sesquiterpene lactones in *Eremanthus* species. The robustness of the method was tested for seven critical operational parameters (extraction time, sample amount, extractor solvent, initial percentage mobile phase, flow rate mobile phase, ELSD temperature and ELSD gain) using eight experiments by PBD [79]. Rechtschaffen design (four factors, 14 experiments) was used for robustness assessment of HILIC method for analysis of granisetron HCl and its related substances [80]. HILIC methods are evidenced to use PBD [81] and FrFD [82] for robustness evaluation.

Youden's test by FrFD has been used to assess the robustness of chromatographic methods to quantify lumefantrine in raw material samples [83], warfarin and its process-related impurities [84]. Patel et al. used FrFD to evaluate robustness of HPTLC methods proposed for simultaneous determination of nadifloxacin, mometasone furoate, and miconazole nitrate [85] and stability-indicating assay of ambroxol hydrochloride and doxofylline [86].

The robustness of an RP-UPLC method for the determination of darifenacin hydrobromide and its related substances was performed using a CCD [87]. A SPE-UHPLC-PDA-FLD method proposed for the determination of cardiovascular drugs in human plasma used eight-experiment (2^{5-2}) FrFD for robustness evaluation [88]. A two-level FFD for four factors [Column temperature (°C), Flow rate (mL min^{-1}), Injection volume (μL), Organic concentration (%)] was used for robustness evaluation of UPLC-ESI/Q-TOF MS method proposed for analysis of rivaroxaban [89]. PBD was extensively employed for robustness evaluation of bioanalytical methods employing HPLC [90,91], UPLC [92], 2D-LC [93] or LC–MS/MS [94] techniques. Kannappan and coauthors employed two-level FrFD [95] and mixture design [96] for robustness tests of enantiospecific HPLC methods for purity determination of chiral switches of pharmaceuticals.

Table 4
Advantages of chemometrics over traditional approaches.

Information	Traditional	Chemometric
Objective/goal	Met by trial and error approach	Attained with predefined method desirability
Performance	Assessed during validation	Dedicated to the method goal
Understanding the method variables	Variables are overlooked	Rational evaluation of individual variables, their interaction and quadratic effect(s)
Quality	Based on method validation	Assured by performance qualification
Qualification, verification and transfer	Are separate exercises	Are continuous exercises throughout life cycle
Regulatory flexibility	No (with respect to changes)	Reduces post-approval changes while working within the design space
Improve	Less	Yes (continuous improvement process)

5.2. Ruggedness

A tutorial to evaluate several noise factors (analyst, instrument or stationary phase batch) was reported to define analytical method ruggedness based on applying design and analysis methodology, combined with risk assessment [97]. A new technique, 'matrix effect map' has been suggested for ruggedness testing of API-LC/MS method. This approach is a graphic presentation of influence of key operational parameters on matrix effects associated with a given method [98]. Hou et al. adapted ruggedness and robustness test to identify potential parameters that influence conversion factors (known or unknown environmental factors) in a SSDMC (single standard to determine multi-components) method for *Salvia miltiorrhiza* determination. Nested-factorial-design was employed to examine factors including different days, analysts, instruments and columns sources [99]. An RPHPLC-PDA method was developed for separation and quantification of 15 carotenoids by applying the isosbestic concept. Three successive ruggedness tests were conducted using 2-level experimental design to establish the robustness of the separation against small variation in composition of the mobile phase, which meets the criteria of relevant European legislation [100]. In a recent study, the optimum sensitivity of a sorbent-based microextraction (fabric phase sorptive extraction) method was evaluated for extraction of penicillin antibiotic residues (benzylpenicillin, cloxacillin, dicloxacillin and oxacillin) from cows' milk by HPLC. An FrFD aided Youden ruggedness test was performed for seven factors (milk mass, stirring during sorption, sorption duration, elution duration, eluent solvent, eluent volume and stirring during elution) to warrant that the method is ready for multi-laboratory testing on multiple instruments [101].

6. Advantages of chemometric approaches

The main objective of chemometry has been to ascertain failure modes and establish robust 'design space', within significant system suitability criteria and uninterrupted life cycle management. The desired state of robust design space here is on the basis of logical multivariate experiments rather than empirical and typical univariate or changing one factor at a time (OFAT) approaches [102,103]. The ubiquitous advantages of chemometrics over traditional approach are well enumerated in Table 4. The potential benefits include: i) better understanding and control over critical variables; ii) beyond traditional approach of method validation; iii) flexibility in analysis of sample in various matrices; iv) improved method robustness thereby reduction of variability; v) analytical attributes within the pharmacopoeial restrictions and away from Out of Specification (OOS) limits; vi) smooth method transfer to the production level; and vii) no obligation of revalidation within the design space.

7. Expanding DOE to quality by design (QbD)

Although DOE application in chromatography gives excellent separation desirability, further method improvements can't be denied. DOE alone can't attain global satisfaction. Recent inclusion of Quality by Design (QbD) initiative by USFDA and ICH (Q8 to Q11) brought revolution to the pharmaceutical community [104]. DoE has evolved to QbD concept. ICH Q8 guidelines defined QbD as "*a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management*" [10]. QbD treatment requires establishing a comprehensive design space as a function of DOE philosophy, within which the global optimal condition locates that provide the desired level of performance of an HPLC method. Implementation of QbD principles allows finding optimal separation conditions for development of robust analytical methods with fewer method transfer or failures issues [4].

7.1. QbD paradigm for LC methods development

Pharmaceutical QbD is a novel approach to the development that begins with predefined objectives and which emphasizes product as well as process understanding and control based on quality risk management. In QbD paradigm, a method is designed to consistently meet its performance desirability criteria; i.e., efficient separation of analytes compliance to regulatory and pharmacopeial requirements. QbD implementation during process/product development undergo through a cycle of five different phases as stated by Rathore and Winkle namely, i) define, ii) design, iii) characterize, iv) validate and v) monitor and control [105]. Fig. 5 describes the key steps involved during the QbD execution in the process or method developments. In the initial step of QbD implementation, analytical target profile (ATP) is identified. ATP is the quality characteristics (e.g., performance or process capability) of an analytical method that ideally will be accomplished to safeguard that desired separation is consistently achieved. This includes, separation efficiency of the method, retention characteristics of the analytes, SST limits etc. Based on the objective of ATP, the critical quality attributes (CQAs) of the method are identified. Subsequent study includes risk analysis via design spaces (DOEs), which allows the analyst to identify the critical method parameters (CMPs) and magnitude of their impact on CQAs. In HPLC methods, the CMPs most often evaluated within the QbD framework by computer modeling softwares (design space) include column temperature, gradient time, aqueous eluent pH and stationary phase. Execution of experiments suggested by DOE enables finding the CMP control strategy to locate critical optimal condition within the design space and afford flexibility during process validation. This facilitates to pave the way for further monitoring and continuous improvement in method performance.

QbD driven computer assisted liquid chromatographic method development has been received global attention for high throughput screening of analytes. Analytical characterization of therapeutic proteins and mAbs by several modes of chromatography (RPLC, IEX,

Table 5
Use of QbD concept in chromatographic techniques (2015–2017).

Method	Analyte	Model/design (DOE)	CMPs	CQAs/ATP	Ref.
HPLC; Stability-indicating	Eberconazole nitrate	3^3 FFD	ΔTBAH (7.5 ± 2.5 mM); Δbuffer pH (2.9 ± 0.3); ΔOrganic phase (25 ± 5 v/v)	Capacity factor	[107]
AEMC; in-silico purification	*Sf*9 insect cell-derived particles	Least-square simulation	ΔpH (7.5–9.0 pH); ΔNaCl (15–120 mM)	Purity; yield; binding strength	[108]
HPLC; Stability-indicating	Fenoverine	(2^{7-3})FrFD (Res IV) for screening; 3^3BBD for optimization	Δbuffer strength (20 ± 10 mM); Δtemperature(25/33/40 °C); Δorganic solvent (81/85.5/90%); Δbuffer type (acetate/formate)	Retention time; tailing factor; number of plates; analytical method volume intensity	[109]
HPLC	Dabigatran Etexilate and related impurities	3^3 FFD; Face centered CCD	Δflow rate (1.0 ± 0.2 ml/min); Δtemperature (30 ± 5 °C); ΔpH (4.5 ± 0.2)	Resolutions between the adjacent peaks; tailing factors; assay	[110]
SFC	Vitamin D3 and related impurities	5^3 CCD; Monte Carlo simulation	Δtemperature (°C); Δgradient time (min); Δethanol proportion (%(v/v)	Separation of critical pair of peaks (7-dehydrocholesterol and 5,6-trans-cholecalciferol)	[111]
UPLC; bioanalytical	Olmesartan medoxomil in rat plasma	3^3BBD for optimization of drug extraction process from plasma. FrFD (2^{5-2}, Res III) for screening; CCD for UPLC optimization	Δextraction time (15 ± 10 min); Δcentrifugation speed (5000 ± 2500 rpm); Δcentrifugation time (10 ± 5 min); Δorganic phase ratio (50 ± 10 v/v); Δinjection volume (1.0 ± 0.5 μL)	%recovery of analyte; Peak area; Theoretical plate; Asymmetry factor	[112]
LC–MS; bioanalytical	Fluoxetine in human plasma	3^3BBD	Δflow rate (0.8 ± 0.2 ml/min); ΔpH (6.7 ± 0.1 pH); Δorganic phase (5 ± 5%v/v)	Retention time; peak area	[113]
HPLC	Five Chemical Components in *Panax Notoginseng* Saponins	Three-level FD; optimal design; Monte Carlo simulation	Δcolumn (Shiseido/Waters/Agilent); Δtemperature (22–26 °C); Δflow rate (0.5–0.8 ml/min). ΔGradient slope (1.6 ± 0.4%/min); initial%B (20 ± 3%); Δinitial isocratic hold (20 ± 5 min).	Analysis time; critical resolutions; peak symmetry	[114]
HPLC	Six alkaloids in Coptischinensis (Huanglian)	PBD; BBD; Bayesian posterior predictive distribution	ΔpH; Δtemperature; Δorganic phase (45 ± 5%); Δsodium dodecyl sulfate (2.85 ± 1.15 g/L); Δpotassium phosphate monobasic (0.03 ± 0.02 mol/L).	Critical resolution; time of analysis; width of peaks	[115]
Achiral SFC	Salbutamol sulfate and related impurities	Rotatable inscribed CCD; parsimonious model; Bayesian model; Monte-Carlo simulation	Δinitial proportion of organic modifier (2–10%); Δgradient time (2–7 min); Δbackpressure (130–170 bar); Δ temperature (35–55 °C)	Retention times at the beginning, the apex and the end of each peak; separation of peaks	[116]
Peptide chromatographic purification procedure	Protein	Ideal cut pooling; 3 different design space borders	Δloading (%); Δgradient duration (%); Flowrate(0.5 ± 2% ml/min); loading (3.27 ± 10% g/L); column length (25 ± 2% cm); saturation capacity (270.7 ± 2% g/L); mass transfer coefficient (61.7 ± 2% min^{-1}); final acetonitrile (39.16 ± 2% %v/v); initial acetonitrile (22.20 ± 2% %v/v); H1 feed concentration (0.0472 ± 10% g/L); L2 feed concentration (0.0429 ± 10% g/L); L1 feed concentration (0.0218 ± 10% g/L)	Yield; productivity	[117]
HIC; Retention Times	Protein	Predictive multivariate models construction and performance evaluation; lsqcurvefit algorithm	Neighbourhood radius; binning (bin size and bin number)	Hydrophobicity (average surface property distribution); Pearson correlation coefficients	[118]
Batch and column chromatography	Natural amino acid histidine	Mechanistic modeling; multiple scale modeling	Ionic capacity per geometric column volume; adsorption isotherms	Histidine capacity; adsorption isotherms	[119]

Table 5 (*Continued*)

Method	Analyte	Model/design (DOE)	CMPs	CQAs/ATP	Ref.
CEX-HPLC; design space modeling; HPLC calibration	mAbs	Hybrid models: mechanistic model (rate model combined with modified Langmuir model; 3 level FFD	Five gradient parameters (solvent composition: %B)	Critical resolutions between main product and the adjacent acidic and basic peaks	[120]
CEX-HPLC; design space determination	mAb and a fusion protein	Model-based high-throughput design (MHD); thermodynamic model; column simulation model and adsorption isotherms; surrogate model	Load pH; elution pH; elution total [Na^+] ($mol.L^{-1}$)	Porosity; radius of a sphere (nm); surface area accessible; resting distance	[121]
UHPLC-UV; UHPLC–MS; AQbD	Protein	PBD; FFD; surface response DOE	Denaturant concentration; denaturing volume; reduction time and temp; alkylation time and temp; Enzymatic digestion temp and time; mobile phase and gradient	% miscleavage; resolution	[122]
LC–MS; peak tracking	Terazosin	DOE	Gradient time; temperature; ternary eluent composition	Retention time; mass	[123]
HAC; purification	mono-PEGylated lysozyme	BBD; Langmuir isotherms	Δgradient length (5–25 column volumes); Δflow (0.8–1.2 $mL.min^{-1}$); Δprotein load (0.25 to 1.75 $mg\,mL^{-1}$)	Yield (%); purity (%); productivity; resolution	[124]
CC	Trimetazidine 2HCl and impurities	CCD; Monte Carlo simulations	ΔpH of water phase (3.5 ± 0.5); Δacetonitrile content (27.5 ± 2.5%); Δconcentration of $HClO_4$ in water phase (150 ± 50 mM)	Retention time (min); Retention factors of first and last eluting peak; selectivity factor between the critical pair of peaks	[125]
UPLC	Triamcinoloneacetonide in hydrogel	CCD; process capability index; Monte Carlo simulation	Δtemperature (30 ± 1.2 °C); Δflow rate (0.4 ± 0.03 ml/min); Δmethanol (53 ± 3%)	Content uniformity; retention factor; percent drug	[126]
UPLC; stability-indicating	Vilazodone HCl	3^3BBD	Δmethanol proportion (85 ± 2%); Δflow rate (1.2 ± 0.1 $mL.min^{-1}$); Δbuffer pH (7.0 ± 0.2)	Plate number	[127]
HPLC	Tamoxifen citrate	TD; BBD	Δmobile phase ratio (55:45 ± 5%); Δbuffer pH (1.0 ± 0.5); Δoven temperature (35 ± 5 °C)	Peak area; retention time; theoretical plates; peak tailing	[128]
RP-LC	Omeprazole	Adsorption isotherm modelling	ΔpH; Δtemperature (°C); type of organic modifier (i.e., acetonitrile or methanol)	Retention factors (omeprazole sulfone& Omeprazole); peak tailings;	[129]
Switch from HPLC to UHPLC; case study	Esomeprazole; omeprazole & impurities	2 level FFD; Continual improvement strategy;	Δbuffer pH (7.5–8.5); Δcolumn temperature (15–25 °C); Δacetonitrile fraction in eluent A (5–15%) and B (75–85%)	Robustness; SST parameters: peak areas; USP tailing; critical resolution; apparent retention factors	[130]
IEMC	mAb	Adsorption kinetic models: first, linear, Freundlich, Langmuir, Temkin isotherms; CADET simulation; mechanistic model	Tubing length; Membrane column length, diameter, porosity; Membrane porosity, radius, column volume, pore size; axial dispersion coefficient; tubing axial dispersion; effective pore diffusivity; External film mass transfer coefficient; Flow velocity; CSTR (continuously stirred tank)	Adsorption and desorption orders	[131]
HPTLC; bioanalytical	Mangiferin in human plasma	D-optimal design; PBD; face-centered cubic design	ΔEthyl acetate (6–8), Δacetic acid (0–2), Δformic acid (1–3), Δwater (1–2); Δvolume loaded (4 ± 2 μl), Δplate dimension (15 × 10 ± 5 × 0 cm)	Retardation factor; peak height; capacity factor; theoretical plates, separation time	[132]
UHPLC–APCI-MS	Coenzyme Q10 and related impurities	Risk based process capability analysis; Monte Carlo simulation	Gradient time (2–5 min); column temperature (30 & 40 °C) at pH 4; linear gradient from 10 to 95% organic modifier	Resolutions; retention of last eluting peak	[133]
HILIC	β-agonists, benzoic acids, nucleosides	QSRR-DOE approach; Partial Least Squares (PLS); CCD; Monte Carlo simulation;	Δacetonitrile content in mobile phase (80 ± 10%); Δwater phase pH (5 ± 2); Δsalt concentration in mobile phase (15 ± 5 mM)	Separation: selectivity factors	[134]

HILIC; stability-indicating	Bilastine and degradation impurities	BBD; FrFD (2^{5-2}); Monte Carlo simulation	ΔAcetonitrile content in the mobile phase (90–94%); ΔpH of the aqueous phase (4.0–5.5); Δammonium acetate concentration in the aqueous phase (40–80 mM/L)	Selectivity factor of critical peak pair; retention factor of the last eluting peak	[135]
RP-HPLC	Cyanidin-3-O-glucoside	BBD	ΔMobile phase ratio (17:83 ± 2%); Δflow rate (0.9 ± 0.1 mLmin^{-1}); Δwavelength (520 ± 10 nm); Δcolumn temperature (40 ± 5 °C)	Retention time; peak area; tailing	[136]
HPLC; stability-indicating	Gliclazide & metformin HCl	FrFD	ΔMobile phase ratio; Δflow rate (0.9 ± 0.1 mLmin^{-1}); Δcolumn temperature (30 ± 5 °C); ΔpH of mobile phase (7.0 ± 0.5)	Resolution	[137]
HILIC; stability-indicating	Olanzapine & related substances	Rechtschaffen design	Δaqueous phase content in mobile phase (7 ± 2%); Δcolumn temperature (45 ± 5 °C); Δduration of linear gradient (14 ± 2 min)	Separation factor for critical pairs of substances (min)	[138]
SPE-HPLC-UV/ELSD	Bioactive components in ShenqiFuzheng injection	PBD; BBD; Monte Carlo probability	Δflow rate (0.3 ± 0.1 mLmin^{-1}); Δcolumn temperature (35 ± 5 °C); Δevaporator temperature (85 ± 25 °C); Δgas flow rate (1.5 ± 0.5 Lmin^{-1})	S/N; desirability for resolution	[139]
2D-HPLC	GNE1 and its related isomer	CCD (face centered); Fusion QBD simulation	Δ%formic acid of MPA (0.1 ± 0.05); Δ%organic in mobile phase (10 ± 5); Δloop size (250, 300, 500 μL)	Resolution; tailing factor; %recovery	[140]
HPLC	Avanafil and dapoxetine	BBD	Δacetonitrile content in mobile phase (60 ± 10%); ΔpH (3.2 ± 0.5); Δflow rate (0.7 ± 0.3 mLmin^{-1}); Δcolumn temperature (20 ± 10 °C)	Peak areas; tailing factors, theoretical plates, and retention times	[141]
SFC	Pharmaceuticals	Three level FFD; global PLS model; retention modeling; LSER descriptors	Δtemperature (16, 35, 53 °C); Δpressure (129, 175, 220 bar); Δgradient slope (3.36, 4.6, 7.37%min^{-1})	Mean apparent retention factors, log P	[142]
HPLC; bioanalytical	Nevirapine in rat plasma	TD; BBD; optimal design	Δmobile phase ratio (64:5:21, 68:9:23, 72:11:25), ΔpH (4 ± 1), Δflow rate (0.75 ± 0.25); Δextraction temperature (°C), Δcentrifugation speed (rpm), Δextraction time (min)	Peak area, retention time, theoretical plates and peak tailing	[143]
HPLC; case study	Saponins from *Panaxnotoginseng*	CCD; Monte-Carlo simulation	ΔPhase B content in elution solution at0 min (13–19 v/v, %); ΔStarting time of the third gradient (6.25–10.75 min); ΔPhase B content in elution solution at the beginning of the third gradient (22.5–31.5 v/v, %); ΔPhase B content in elution solution at 11 min (32.5–41.5 v/v, %)	Resolutions of three critical peak pairs	[144]
SFC; enantioseparation	Melatonin, agomelatine and naphtalen derivatives	CCD (circumscribed)	Δoutlet pressure (80–200 bar); Δflow-rate of the mobile phase (2–5 mL/min); Δpercentage of ethanol as co-solvent (10–30).	Resolution; analysis time	[145]

Table 5 (*Continued*)

Method	Analyte	Model/design (DOE)	CMPs	CQAs/ATP	Ref.
RP-HPLC; in silico robustness test	Methylparaben sodium, propylparaben sodium, hydrocortisone, fexofenadine HCl, ibuprofen and pioglitazone	FFD; retention model; error propagation modeling	Δbuffer pH (3.0 ± 0.6); Δternary blend ratio (acetonitrile/methanol); ΔGradient time at 5 and 15 min	Retention factors; iterated retention times of all peaks	[146]
HPLC; enantioseparation	BEL097, BEL174, BEL169	CCD	ΔColumn temperature (10–25 °C); Δpercentage of trifluoroacetic acid (0.07–0.15) and Δproportion of n-hexane in mobile phase (3–10%)	Resolution	[147]
RP-LC; stability-indicating	Dabigatran etexilatemesilate and impurities	BBD; Monte Carlo simulations	Δacetonitrile content at the start of gradient program (22 ± 2%); Δacetonitrile content at the end of gradient program (57 ± 3%); Δgradient time (10 ± 2 min)	Critical separation: retention time of impurity peak beginning and end	[148]
UHPLC–MS; impurity profiling	Sumatriptan and naproxen	FrFD; Monte Carlo simulations	Δflow rate (0.3 & 0.4 mL/min); Δtemperature (30 & 40 °C); Δgradient time (10 & 15 min)	Number of peaks failure to exceed specific resolution	[149]
UHPLC; inter-batch column repeatability	Amlodipine and impurities	Design space (Dry Lab® models); peak tracking	Δgradient time and range; Δtemperature; ΔpH	Critical resolutions; robustness (% of successful experiments with $R_{s,crit} > 1.5$)	[150]
UPLC/Q-TOF-ESI–MS/MS; impurity profiling	Imatinib mesylate and related impurities	BBD; Dry Lab® modeling	Δgradient time; Δtemperature (42 ± 5 °C); ΔpH (8.0 ± 1.0); Δflow rate (0.5 ± 0.05 mLmin^{-1}); wavelength (237 nm, 254 nm, and 269 nm)	Critical resolutions; critical tailing	[151]
UHPLC; impurity profiling	Dextromethorphan HBr and degradation products	PBD; CCD; Derringer's desirability; greenness score	Δflow rate (0.20–0.40 mL/min); Δtemperature (30–50 °C); ΔpH (2.0–6.0); Δgradient slope (2.0–4.0%/min)	Resolutions; theoretical plates; ethanol volume; mobile phase volume	[152]
HILIC; stability-indicating	Iohexol and impurities	PBD; BBD; Monte Carlo simulation	Δacetonitrile content in the mobile phase (85 ± 5%); ΔpH of the water phase (5.0 ± 2.0); Δammonium acetate concentration in the water phase (50 ± 30 mmol L^{-1})	Retention of analytes (retention time, min)	[153]

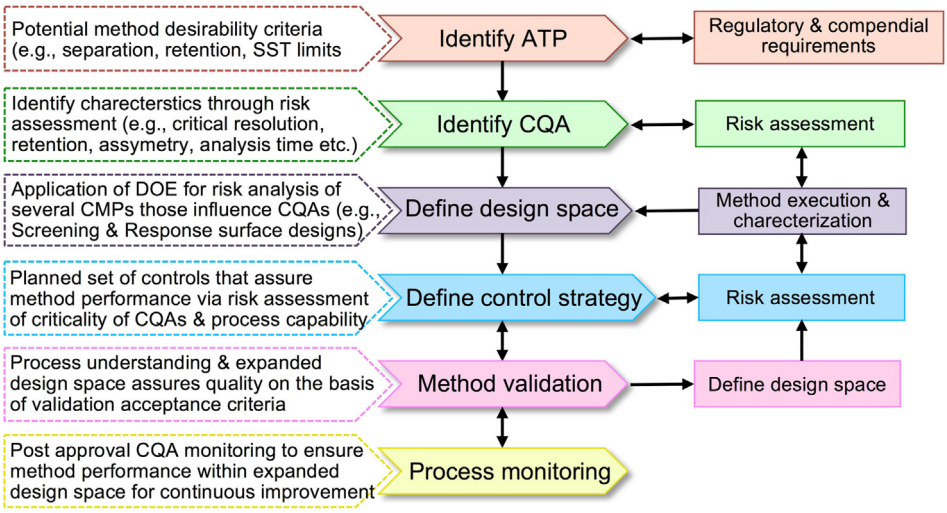


Fig. 5. Key steps in QbD driven chromatographic method development.

HIC) has been critically reviewed for most important CMPs (critical variables) [106]. Many examples have been proven that HPLC method development and validation is incomplete without QbD (Table 5).

8. Six-Sigma practice in chromatography

Six-Sigma is a set of techniques and tools for process improvement associated with statistical modeling of the processes. Originally, it referred to the ability of manufacturing processes to produce a very high proportion of output within specification. A sigma rating is used to indicate the process yield in terms of the percentage of defect-free outputs. A Six-Sigma process is one in which 99.99966% of all outputs of a process are statistically expected to be free of defects, that is tantamount to say that there may be 3.4 noncompliant outputs per million opportunities. The International Organization for Standardization (ISO) has published in 2011 the first standard "ISO 13053:2011" defining a Six-Sigma process. Sigma metrics is a quality indicator. For quantitative assays, before calculating the sigma value, imprecision (%CV) and inaccuracy (%bias) have to be evaluated. The latter can be obtained comparing the value obtained in the lab with a specific method to the value obtained using a reference method, according to the following Eq. (16):

$$\%bias = \frac{lab\ value - reference\ value}{reference\ value} \times 100 \quad (16)$$

The sigma number can be calculated according to the following equation that describes the relationship between imprecision and inaccuracy to Sigma metrics Eq. (17).

$$Sigma\ metric = \frac{TAE - bias}{CV} \quad (17)$$

Where, TAE is the Total Allowable Error.

As process coefficient of variation goes up, or the inaccuracy increases, fewer standard deviations will fit between the mean and the nearest specification limit, decreasing the sigma number and increasing the likelihood of items outside specification. It is important to strive for at least 6-Sigma even if results above 3-Sigma are acceptable. The chromatographic process has characteristics that can be defined, measured, analyzed, improved, and controlled; hence it may be amenable to Six-Sigma overall process control. Glycated Haemoglobin (HbA1c) assay is the cornerstone of diabetes care. The International Federation of Clinical Chemistry Task Force on Implementation on HbA1c Standardization endorsed the sigma metric for setting quality targets within and between laboratories. The analytical verification and quality assessment of the recently introduced, user-friendly, and fully automated IE-HPLC Tosoh HLC-723GX HbA1c analyzer represent an interesting case study to show the use of these methods [154]. Lyophilized samples (n = 5) were used for the assessment of bias calculated according to the reference method based target values. At the selected HbA1c clinical decision level the bias was 0.04%, imprecision was 0.04% and the Six-Sigma analysis gave σ value of 3.91, within the desirable classification of performance and the recommended sigma-based quality criteria for routine laboratories.

The Lean Six-Sigma (LSS) methodology emphasizes the importance of eliminating all kinds of waste. The LSS methodology focused on waste management was applied in developing a new reverse phase liquid chromatographic method for the estimation of Ranitidine HCl from tablet formulation [155], to reduce the organic solvent and for the improving of an HPLC method for the estimation of Levothyroxine Sodium in tablet formulation [156]. In both cases Define, Measure, Analysis, Improve and Control (DMAIC) principles were used for problem solving, root cause investigation, risk management to improve method performance. The HPLC analytical procedure was subjected to a Failure Mode and Effects Analysis (FMEA), including technical risks as well as risks related to human failure. An FMEA tool broke down the HPLC analytical method into process steps and identified possible failure modes for each step. The HPLC method was broken down into steps; possible failure modes for each step were identified. Each failure mode was ranked on the basis of estimated frequency of occurrence (O), probability that the failure would remain undetected later in the process (D) and severity (S), each on a scale of 1–10. Risk Priority Numbers (RPNs = O × D × S) were estimated. Failure modes with the highest RPN scores were subjected to corrective actions and the FMEA was repeated. In both cases the investigations showed that it was feasible to define an HPLC method with an improved quality compared to official (IP2010) assay method.

9. Future prospectives

Chemometry, since its origin, has been experienced disparate band of enthusiasts and now became mature enough as a discipline for the community to readdress. With the advent of scientific computing, several applied informatics and computing disciplines like: multivariate calibration; *t*-statistic; *F*-statistic, Six-Sigma practice,

ANOVA and principal component analysis (analytical chemistry); experimental design (industrial chemistry); bioinformatics (quantum chemistry); chemoinformatics; multiway analysis, artificial neural networks and artificial intelligence became integral parts of chemometrics. More studies on advanced bioanalytical techniques using liquid chromatography are needed to discover biomarkers of infections useful for rapid diagnosis of diseases. To evidence the index of clinical suspicion, large number of samples pooled from patients over different geographical regions requires flawless data analysis or experimental design. Hyphenated techniques with improved ionization, more sensitive MS parameters are crucial resources in this respect. A recent study demonstrated the increasing popularity of chemometrics practice and publications in core scientific journals. An estimated average of around 6000 papers per year have some recognizable chemometrics content even if it was not able to capture large funds and to develop a solid academic base in western countries [157]. Nevertheless, its use is going viral, hence, in near future core chemometrics will come up as a new area of discipline in academics with enormously encouraged industrial grants.

10. Conclusions

Raw experimental data would be meaningless without models able to provide meaning from observable fact. In chromatography, the extended optimization parameter space (pH, type, and lipophilicity of additives, organic modifier concentrations, ionic strength, stationary phase packing etc.) and analyte differences (non-ionic, ionizable, and ionic solutes) may make the rational selection of optimal experimental conditions a major challenge in the development of a chromatographic method. At variance with theoretical models that follows from known theoretical laws or principle and are predictive in their own right chemometric models are empiric but very versatile and useful to describe the system response [158]. Chemometrics is certainly a sector of increasing and brilliant perspective for the future of analytical chemistry. Some of the main aspects of chemometry and its applications in HPLC method development and validation have been described. The valuable information it provides from some reported experiments has also been discussed. A chemometric (multivariate) approach can be used to rigorously assess all types of variables of an analytical method rather than using generic “changing one variable at a time” practice, since it is economical, less time consuming and require less number of experiments. Several case studies were exploited for selection of type and number of factors including their levels, appropriate design, software platforms and statistical interpretation of factorial effects based on the method objectives. Interestingly, the chemometric approach is valuable also for sample preparation, stability indicating methods, and other miscellaneous chromatographic techniques. Optimization of the HPLC separation of the analytes still play a major role notwithstanding the progressive substitution of traditional detectors with multiple MS instruments whose parameters are however amenable to chemometrics optimization too. The applicability of chemometrics in various validation parameters (precision, ruggedness, robustness, system suitability), which are essential prerequisites to ensure the methods’ reliability for routine use, has been outlined. Setting-up an appropriate experimental design maximizes the discoverability, estimation and control of any sources of variability and enables to employ necessary strategies to improve method performance.

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