Optimization of PEG–salt aqueous two-phase systems by design of experiments

Anna Glyk, Dörte Solle, Thomas Scheper and Sascha Beutel

Institute of Technical Chemistry, Leibniz University of Hannover, Callinstr. 5, 30167 Hannover, Germany

*To whom correspondence should be addressed:
E-mail: beutel@iftc.uni-hannover.de. Phone: + 49-511-762-2868. Fax: + 49-511-762-3004

Type of Article: Review
Abstract

Since the mechanism governing the partitioning behavior of biomolecules, such as proteins and enzymes, in polyethylene glycol (PEG)–salt aqueous two-phase systems (ATPS) is complex and not easily predictable, many laborious experiments have to be performed for an optimization of these systems, causing increased overall cost. However, the multivariate statistical design of experiments (DoE) methodology is representing a promising and efficient optimization technique which can overcome the limitations of traditional optimization methods. Therefore, DoE has emerged as a powerful and efficient optimization tool for PEG–salt ATPS, since it is faster, more efficient and cost-effective, allowing a simultaneous and rigorous evaluation of process/system parameters. In the present review, different DoE process steps are represented to highlight the feasibility of this approach to operate as a promising and efficient optimization tool, thus facilitating the evaluation of the partitioning behavior, recovery and purification of different proteins and enzymes in PEG–salt ATPS. In this context, several experimental designs, such as factorial and response surface designs, have been discussed and evaluated by statistical regression analysis and analysis of variance (ANOVA), as well as various applications of PEG–salt ATPS using DoE have been outlined which may further promote the optimization of these systems.

Keywords: Aqueous two-phase systems; Design of experiments; Factorial experimental design; Response surface methodology; Central composite design; Analysis of variance
Contents

1. Introduction ....................................................................................................................................... 4
2. Theory and methodology .................................................................................................................. 6
   2.1 Screening of significant factors ........................................................................................................ 6
   2.2 Steepest ascent / descent method ..................................................................................................... 9
   2.3 Optimization by RSM ...................................................................................................................... 9
   2.4 Analysis of model ............................................................................................................................ 14
   2.5 Validation of model .......................................................................................................................... 20
3. Applications of PEG–salt ATPS using DoE ....................................................................................... 20
4. Conclusions ........................................................................................................................................ 23

References ............................................................................................................................................. 25
1. Introduction

Since partitioning, recovery and purification of biomolecules, such as proteins and enzymes, in polyethylene glycol (PEG)\textsuperscript{1}salt aqueous two-phase systems (ATPS) are influenced by several system parameters and physicochemical/surface properties of the target biomolecules, the mechanism governing the partitioning behavior of biomolecules in ATPS is complex and cannot be easily predicted [1–6]. Thus, many experiments have to be performed for the optimization of ATPS which is tedious, leading to increased overall cost [7]. One conventional optimization method is the one-factor-at-a-time (OFAT) approach consisting of a chosen starting point or baseline set of levels for each factor, the determination of significant process/system factors and a consecutive change of each factor at a time over a certain range by keeping all the other factors constant at the baseline level [7–9]. The major disadvantage of this approach is that possible interactions between the factors are not considered, and hence no combined effects of all factors are depicted [9, 10]. Thus, OFAT experiments are often unreliable, usually leading to poor results or false optimal conditions/factors determined for a process/system, and this method is generally inefficient, time-consuming and laborious, since many experiments are required [7–9, 11]. Nowadays, the optimization of partitioning processes in PEG–salt ATPS is widely carried out by a multivariate statistical technique called design of experiments (DoE) which is based on a statistical factorial experimental design concept, consisting of the performance of a few experiments at a particular factor level combination [7, 9, 12].

\textsuperscript{1}Abbreviations used: PEG, polyethylene glycol; ATPS, aqueous two-phase systems; OFAT, one-factor-at-a-time; design of experiments, DoE; RSM, response surface methodology; FFD, full factorial design; fFD, fractional factorial design; ANOVA, analysis of variance; PBD, Plackett–Burman design; CCD, central composite design; CCC, central composite circumscribed; CCF, central composite face-centered; BBD, Box–Behnken design; MLR, multiple linear regression; F-test, Fisher's statistical test; t-test, Student's t-test; p-value, probability value; SS, sum of squares; LOF, lack-of-fit; MS, mean squares; DF, degrees of freedom; SOR, significance of regression; $R^2$, coefficient of determination; $R^2_{\text{adj}}$, adjusted coefficient of determination; $Q^2$, coefficient of predicted variation; $K$, partition coefficient; $Y$, product recovery/activity yield ($\%$); $PF$, purification factor; $S$, selectivity; $P$, purity ($\%$).
In general, experiments are used to investigate the performance of a process/system [9]. Thereby a process/system (Fig. 1) can be generally described as a combination of methods, operations, machines, people and other resources inducing a transformation of inputs (often materials) into outputs [9, 13]. The measured responses $Y_1, Y_2, \ldots, Y_n$ are used to characterize the performance/quality of an investigated process/system, providing information about its properties and general conditions [13]. On the other hand, factors are representing tools for the manipulation of a process/system [13]. By altering the most influential factors, the process/system features might be changed according to a desired response profile [13], at which some of the factors $X_1, X_2, \ldots, X_n$ (e.g., temperature) are controllable, whereas other factors $Z_1, Z_2, \ldots, Z_n$ (e.g., climate) are uncontrollable [8, 9]. The performance of a designed experiment is usually related to the determination of the effects of changed controllable input factors ($X_1, X_2, \ldots, X_n$) on the corresponding varied output responses ($Y_1, Y_2, \ldots, Y_n$) of a process/system [8, 9, 13].

→ Fig. 1

Since all variables involved in a process/system have a significant influence on the experimental performance, a designed experiment may include the following objectives according to [8, 9]:

1) Determination of the most influential variables on responses $Y_1, Y_2, \ldots, Y_n$.

2) Determination of optimal settings of influential $X_1, X_2, \ldots, X_n$ resulting in $Y$ which is almost always near the desired nominal value.

3) Determination of optimal settings of influential $X_1, X_2, \ldots, X_n$ resulting in a small variability in $Y_1, Y_2, \ldots, Y_n$.

4) Determination of optimal settings of influential $X_n$ minimizing the effects of uncontrollable factors $Z_1, Z_2, \ldots, Z_n$.

Moreover, several significant factors can be changed and optimized simultaneously in the multivariate DoE approach in contrast to the OFAT method [7, 9]. Therefore, the DoE
approach is faster, more efficient and cost-effective for a rigorous evaluation of the significant factors on the selected responses and their possible interactions using a mathematical model (usually a quadratic/second-order or higher polynomial function) [13], thus overcoming the limitations of the OFAT method. Overall, a DoE process for the optimization of PEG–salt ATPS is consisting of the following steps:

1) Screening of significant factors
2) Steepest ascent/descent method
3) Optimization by response surface methodology (RSM)
4) Analysis of model
5) Validation of model

All of these steps are highlighted in Fig. 2 and discussed in the following chapters.

→ Fig. 2

2. Theory and methodology

2.1. Screening of significant factors

Initially, in a DoE process for the optimization of PEG–salt ATPS a screening of a large number of factors $k$ is carried out in a few experiments in order to reveal the most important factors having a statistical significant influence on the output responses or performance of a process/system and investigate their appropriate ranges [7, 8, 13, 14]. Furthermore, the purpose of a screening design is the identification and selection of those factors demanding a more thoroughly investigation in further experiments [8, 9]. In general, screening of significant factors in PEG–salt ATPS is widely carried out by the full factorial design (FFD) and fractional factorial design (fFD). These experimental design approaches are generally performed by assigning all factors $k$ at two-levels, usually denoted by a high (+1 or simply +) and low (−1 or just −) level for each factor [7–9, 13]. These notations are often called the
orthogonal/effect coding and are consequently used to construct an orthogonal arrangement or design of experiments [9, 13].

The two-level FFD is carried out at all possible factor level combinations for all factors and is consisting of a set of experimental runs with a total number of $2^k$ [7–9, 13]. For instance, the $2^2$ FFD for two factors (e.g., A and B), is resulting in four possible experimental runs at the four factor combinations: $(-, -), (+, -), (-, +)$ and $(+, +)$, according to the first four rows in the design matrix in Fig. 3a [7–9, 13]. Furthermore, this design can be described as a square containing an experimental region of a regular geometry (Fig. 3b), at which each row in the design matrix is corresponding to one experiment depicted as a point in the two-dimensional factor space [13]. In addition, a $2^3$ FFD for three factors (e.g., A, B and C) can be designed similarly to the $2^2$ FFD using high (+) and low (−) factor levels leading to eight possible experiments at eight different factor combinations, as summarized in the eight rows in the design matrix in Fig. 3a, while the corresponding experimental region can be illustrated by a cube of a regular geometry (Fig. 3c) [9, 13].

Furthermore, the interaction effects among factors on a response can be determined in FFD’s by a simultaneous alteration of factor levels, at which a linear response over a selected factor level range is assumed in these designs because each factor has only two-factor levels [7–9]. However, with five or more factors in a $2^k$ factorial design the number of experiments is drastically increased, resulting in a fast outgrowing of the resources of most experimenters in terms of too demanding experiments [7–9, 13]. Hence, the two-level fFD is usually used as a more appealing experimental screening design which is based on the FFD and may be constructed by choosing only fractions of corner experiments [8, 13–15]. Generally, the fFD is denoted by $2^{k-1}$, $2^{k-2}$ and $2^{k-4}$ containing a total number of experimental runs reduced to a one-half, one-quarter or a higher fraction of the FFD [7–9, 13]. For instance, a $2^{3-1}$ fFD is resulting in four experimental runs of three factors (e.g., A, B and C), as shown exemplarily in
a cube of a regular geometry with coded units in Fig. 3d, according to the rows 1, 4, 6 and 7 in
the design matrix in Fig. 3a [9, 13].

Moreover, FFD and fFD usually include 3–5 replicated center-point experiments which are
carried out for an evaluation of the pure experimental error [9, 13, 15–18]. These replicated
experiments are called center-points because they are performed in the center of the
experimental region located between the high and low levels, thus increasing the factor levels,
at which the center level is denoted by 0 in a design matrix [13]. Hence, center-points
characterize experimental runs at the center level of each factor range [6, 19]. Furthermore,
the pure error of the replicated center-point experiments is used to estimate the statistical
significance of the calculated values of each factor, and thus evaluate the background
variability of the process [6, 19, 20].

Another screening design method sometimes used in PEG–salt ATPS is the orthogonal two-
level Plackett–Burman design (PBD) when only main influences are of interest, assuming that
no interactions between different factors \( x_i \) are considered in the range of variables, thus
considering a linear screening approach [7, 10, 13–15, 21]. Since the PBD is a non-geometric
design consisting of irregular fractions of \( 2^k \) designs and can be designed with a multiple
number of four experimental runs, this screening design is consequently used for irregular
experimental regions according to inaccessible corner experiments [7–9, 13].

Overall, since the described experimental screening designs represent linear approaches,
they are based on a first-order model according to eq 1 [9, 10, 15, 16].

\[
y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \epsilon
\]

Here, \( y \) is the predicted response variable, while \( \beta_0 \) and \( \beta_i \) are the regression coefficients of
the model. Furthermore, \( x_i \) is a variable representing an experimental factor and \( \epsilon \) is a random
error. Finally, the statistical significance and magnitude of the input factors in all three
screening designs can be generally analyzed and interpreted by using the analysis of variance.
(ANOVA) in order to calculate and evaluate the main effects of each factor and all their possible interactions on the output response variables [7, 9, 22].

→ Fig. 3

2.2. Steepest ascent / descent method

After determination of significant factors it is necessary to confirm that these factors and the output responses are located in the vicinity of the optimal experimental region which is performed by an analysis of the model curvature [7, 12, 14]. In this context, a model curvature occurs if there is a significant difference between the average output responses of the screening design and center-point experiments at a confidence level of 95 % evaluated by ANOVA [7, 13, 14]. That means, the output responses are situated in the vicinity of the optimal experimental region, thus the significant factors can be optimized by RSM in the next DoE step [7, 14]. On the other hand, when the output responses are far away from the optimal experimental region no difference or curvature is existing, and therefore steepest ascent [16, 18, 23, 24] or steepest descent [25] experiments have to be carried out in order to increase or decrease the output responses, respectively, thus reaching the proximity of the optimal experimental region and investigate the experimental direction [7, 9]. These steepest ascent/descent experiments are initially performed at the center-point of the significant factors and each factor level is increased or decreased according to the magnitude of the main effects [7]. Furthermore, these experiments are carried out until the output responses cannot be further increased, thus reaching the maximal output response points assumed as a general vicinity of the optimal experimental region, and finally these points can be used as center-points for the optimization by RSM in the next DoE step [7].

2.3. Optimization by RSM
The next step in a DoE process is the optimization of the significant factors by means of response surface methodology (RSM) [7, 14]. This approach was first reported by Box and Wilson [26] and has proved to be a very efficient optimization tool [27]. In recent years, the use of RSM has gained importance in the evaluation of biotechnological processes and has become an innovative method in several research studies [17, 28, 29]. Furthermore, this approach is used as an effective statistical technique in order to determine the optimal operating conditions and significant independent factors or their interactions with the dependent output responses in multivariate complex systems, such as PEG–salt ATPS, thus enhancing the output responses for the optimization of these systems considering the partitioning of biomolecules, and consequently designing complex processes occurring in ATPS [2, 11, 17, 19, 30–32]. Moreover, RSM is a useful and promising tool for establishing a statistical model to predict output responses or partitioning target parameters of ATPS by a correlation of the measured output response factors to the input factors, thus modelling a process, such as the separation and purification of biomolecules in ATPS [2, 32–36]. Therefore, it is common to investigate an optimal experimental region and obtain experimental data, providing usually a quadratic/second-order polynomial model fitting for the prediction of responses which are analyzed and validated in the next DoE steps [7, 9, 37]. In this context, a multiple regression analysis of the experimental data is performed to evaluate the optimal mathematical model equation, at which target output responses can be analyzed by response surface or contour plots as outlined separately ahead [7, 10, 18, 34, 38]. In other words, the RSM approach is used for a verification of a regression model prior to its transformation to response surface or contour plots [13]. Thereby, it is based on different multilevel designs, such as the central composite and Box–Behnken design [7, 9, 13].

The central composite design (CCD) is the most frequently used design among RSM designs for fitting quadratic/second-order polynomial models and is characterized by orthogonality, rotatability and a uniform precision [9, 13, 30, 32, 35]. Furthermore, this design
is extensively utilized for the optimization of PEG–salt ATPS. In general, the CCD is based on two-level FFD or fFD and can be described as a natural extension of these designs [13]. Furthermore, it is composed of the following three building blocks according to [9, 13, 19]:

1) Regular arrangement of corner experiments of a two-level factorial design ($2^k$ FFD or $2^{k-1}$ fFD)

2) Replicated center-points

3) Symmetrical arrangement of star/axial points located on factor axes.

Thereby, star/axial points are representing the main difference between factorial and central composite designs [13]. Furthermore, the CCD can be generally divided into central composite circumscribed (CCC) and central composite face-centered (CCF) designs according to the position of the star/axial points [13]. Moreover, these designs are differing in the characteristic parameter $\alpha$, representing the distance from the star/axial points to the center-point, at which $\alpha$ is primarily selected according to the experimental region of interest, while the factor level coding is depending on the chosen design and value of $\alpha$ [9, 11, 32]. Hence, the value of $\alpha$ determines the type of design and makes it flexible [39].

The CCC design circumscribes experimental runs by star/axial points located at a level outside of the low and high factor settings of the composite design, thus facilitating curvature modelling and investigating each factor at five levels [6, 11, 13, 19, 30]. For instance, the CCC design for three factors (e.g., $x_1$, $x_2$, and $x_3$) is containing eight corner experiments, six axial experiments and, at least, three replicated center-points (Fig. 4a) [9, 13]. Since this design is providing a curvature modelling or second-order model, good predictions throughout the experimental region of interest are facilitated by a rotatable second-order response [9, 40]. In this rotatable CCC design $\alpha$ has been set to $\pm 1.68$ [14, 32, 41, 42] and $\pm 2$ [19, 33, 43–45] for the optimization of PEG–salt ATPS. Moreover, since the rotatability is a spherical feature, a spherical experimental region of interest has to be evaluated, in which all factorial and axial points are located on a spherical surface [9]. In this spherical CCC design $\alpha$
has been frequently set to $\pm 1.41$ [6, 18, 23, 35, 38, 46–49] for the optimization of PEG–salt ATPS. Hence, the CCC design for three factors is obviously represented by a hyperspherical experimental arrangement due to corner and axial experiments approximating a spherical surface (Fig. 4a) [13]. On the whole, the CCC design can be described as a rotatable or spherical design according to the experimental region of interest.

However, when the experimental region of interest is cuboidal rather than rotatable/spherical or when it is preferable to maintain low and high factor levels, and still carry out a RSM design, the CCF or face-centered cube is a feasible alternative involving $\alpha = \pm 1$ [9, 13], which is commonly used for the optimization of PEG–salt ATPS [2, 16, 17, 22, 25, 28, 31, 34, 36, 49–57]. For instance, in the CCF design for three factors (e.g., $x_1$, $x_2$, and $x_3$) the star/axial points are located on the centers of the faces of a cube or hypercube (Fig. 4b), thus all factors have three levels rather than five and the experimental region is representing a cube/hypercube instead of a sphere [9, 13]. This design is recommended for full scale and pilot plant investigations [13]. Furthermore, since the CCF design is requiring only three factor levels, it is a beneficial design because a change of factor levels is usually difficult in practice [9]. However, the CCF design is not rotatable and the CCC design is generally favored in comparison to the CCF design as it is spanning a larger volume at the same given low and high factor level settings leading to a better design for capturing a strong curvature owing to its five factor levels, even providing a cubic modelling behavior [9, 13]. On the other hand, the CCC design has less correlated quadratic model terms than the CCF design which is advantageous in terms of a regression modelling [13]. Overall, RSM designs (e.g., CCC, CCF) are usually used for the evaluation of systems involving two to five factors [13]. In this context, a tabulated overview of the required experimental number in these designs is represented in Fig. 4c containing additionally the factors six and seven for comparative purposes [13]. However, with a raising number of factors to six or more, the experiments are
strongly increased in these designs leading to a less feasible RSM, and therefore other alternative designs have to be employed [13].

→ Fig. 4

The Box–Behnken design (BBD) is an alternative experimental design for RSM sometimes used for the optimization of PEG–salt ATPS which is providing only three-levels (+1, 0, −1) for each varied factor, as shown in the BBD matrix in Fig. 5a for three factors (e.g., $x_1, x_2, x_3$) [9, 10, 13, 15, 58, 59]. This design is a rotatable/spherical or nearly rotatable second-order design based on a three-level partial or incomplete factorial design consisting of the middle points of the edges from a cube and the center-point, as represented in Fig. 5b for three-factors (e.g., $x_1, x_2, x_3$) [9, 13, 15, 39, 59]. Hence, this design can be considered as three interlocking $2^2$ factorial designs along with a center-point [39]. Moreover, although the BBD can be derived from a cube, it is a spherical design, i.e., the vertices of the cube are not covered by this design, and therefore, prediction in these points is an extrapolation and should be avoided [39]. The BBD is a beneficial design when the corner experiments are undesirable, extremely expensive or impossible to analyze due to physical process conditions [9, 13]. Mostly, this design is used for the investigation of three or four factors [10, 15, 59] and does not exist for two factors [13]. For instance, the BBD is requiring a less number of experiments for three factors in comparison to the CCD (15 vs. 17), as shown in Figures 4c and 5a, respectively, and it is beneficial compared to the CCC design, as it has usually less factor levels (3 vs. 5).

However, CCD or CCC/CCF designs are usually favored in optimization studies owing to the fact that they are based on full and fractional factorial designs [13]. Since screening is often initiated by FFD/IFFD, an optimization is usually performed by using CCD or CCC/CCF designs because they are more tractable, so that screening designs can be easily converted into CCC/CCF optimization designs [13]. Furthermore, the CCD is an effective design usually not requiring many experimental points, and consequently reducing the overall cost [51, 60].
Generally, the main advantage of RSM is a decreased number of experimental runs required for estimation of multiple factors and their interactions [51, 61]. Finally, the results or experimental data of this approach are used for fitting a quadratic/second-order polynomial model statistically evaluated by regression analysis [7], as discussed in the next chapter.

→ Fig. 5

2.4. Analysis of model

After screening and optimization of significant factors the obtained mathematical model has to be analyzed for each of these DoE steps, as outlined exemplarily for the quadratic/second-order polynomial equation resulting from optimization. For that purpose, the obtained quadratic/second-order polynomial equation for predicting optimal values in PEG–salt ATPS has to be solved analytically by a statistical regression analysis or response surface regression procedure according to eq 2 [7, 10, 16, 34, 51, 62, 63].

\[
y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} \beta_{ij} x_i x_j + \epsilon \quad (i \neq j)
\]

Here, \(y\) is the predicted response variable, \(\beta_0, \beta_i, \beta_{ii}\) and \(\beta_{ij}\) are the regression coefficients of the intercept, linear, quadratic and interaction \((i \neq j)\) effects, respectively, while \(x_i\) and \(x_j\) are independent input variables, and \(\epsilon\) is a random error. Moreover, eq 2 can be analyzed graphically by using three-dimensional response surface plots or contour plots in order to evaluate the optimal values [7]. An analysis of these plots is providing a method to reveal the interactions between two significant factors, all their combinations and one studied response variable in order to determine the most efficient conditions for an optimal target response variable [6, 10, 14, 16, 19, 37, 51, 64]. Thereby, three-dimensional response surface plots indicate the effects of operating factors on a response variable, while contour plots represent the nature and magnitude of these effects [15]. In addition, several time-saving and useful statistical software packages are summarized in Table 1, which are used in terms of
analysis and performance of statistical model calculations [7, 9]. The first three software packages (Design-Expert, Minitab and MODDE) are recommended for scientists who are new to the field of DoE since these software packages are facilitating an intuitive operating without any knowledge regarding programming, whereas the last three software packages (SAS, SPSS and STATISTICA) are recommended for scientists who are familiar with programming.

→ Table 1

The statistical significance of quadratic models or equations and their corresponding coefficients is generally evaluated by the analysis of variance (ANOVA) and standard least squares techniques [9, 15, 22, 37], at which a multiple linear regression (MLR) analysis is usually performed in order to identify the coefficients of the significant effects of each response variable [22, 53]. In this context, ANOVA is carried out for the determination of the significance and magnitude of the effects of variables and all their possible linear and quadratic interactions on a studied response variable by using Fisher's distribution or Fisher's statistical test (F-test), as discussed separately ahead [22]. In general, models/equations are validated by a statistical procedure, at which the statistical significance of all effects on the model or their corresponding regression coefficient parameters with less than 95 % significance (95 % confidence level) are initially considered as non-significant according to the Student's t-distribution or Student's t-test (t-test) and the probability of error or probability values (p-values), i.e., \( p > 0.05 \) [10, 17, 19, 22, 34, 43, 45, 49]. Hence, these effects or parameters are excluded and added into the residual error term and a new ANOVA is performed for the complete and reduced model [22, 37, 38, 45, 52]. That means, the new reduced models are obtained for response variables by using only significant factors in a regression analysis [56], at which the \( p \)-value, defined as the smallest level of significance leading to the rejection of null hypothesis, is smaller than 0.05 (\( p < 0.05 \)), which means that a particular model term is statistically significant at 95 % confidence level, thus representing a
decreasing index of the reliability of a result [15, 22, 33, 51, 65]. In other words, ANOVA is used as an important statistical tool in the MLR analysis based on the identification of a regression model, in which the residual sum of squares of the response variable is reduced [13]. While dealing with designed data, addition and removing of terms from an MLR model is feasible, thus creating revised models [13]. ANOVA and other statistical tools are performed in order to evaluate and compare revised models with the originally predicted model in terms of model adequacy, thus providing a method for the evaluation of an alternative model performance [13]. Since a common term in ANOVA is the sum of squares (SS), which is useful to quantify the variability and can be distributed into smaller components, ANOVA is based on the distribution of the total variation of a response variable into one component related to the regression model and another part referring to the residuals [13]. Furthermore, when replicated experiments (e.g., replicated center-points) are available, the residual variation is distributed by ANOVA into one part referring to the model error and another part related to the replicate error [13].

Subsequently, the numerical sizes of variances or their estimations and the statistical significance of the obtained models and excluded parameters can be compared to each other and evaluated by performing the $F$-test for a set of parameters or output data set considering either $F$- or $p$-values of the model and the lack-of-fit (LOF) [13, 22, 53]. In this context, another common term in ANOVA is the mean squares (MS), which can be determined by the ratio of SS to the corresponding degrees of freedom (DF) [43]. Furthermore, $F$-values are defined as the ratio of the mean squares of the regression error of the model to the mean squares of the residual error term, while $p$-values can be estimated from the $F$-distribution or $F$-table, in which the critical limit of $p$ is commonly set to $p = 0.05$ [13, 22, 43, 52]. Furthermore, the $F$-distribution is providing the $F$-values and the corresponding DF of the model and the respective error term [43]. Hence, the computed $p$-values from the $F$-distribution are used in order to evaluate the significance of each coefficient, which is
necessary in terms of understanding the pattern of the mutual interactions [43]. Thereby, the smaller the magnitude of the \( p \)-values, the more significant is the corresponding coefficient [43]. On the other hand, the LOF test, providing a comparison between the residual error (i.e., error associated with the fitted model) and the replicate error, is used in order to determine the significance of the predicted model [43]. In case of the LOF test, the \( F \)-values are determined by the ratio of the mean squares of the model or regression error to the mean squares of the replicate error instead of the residual error [13, 43]. Generally, two variance pairs are compared in the \( F \)-test which is consisting of two parts [13]. The first \( F \)-test, comparing the variation in the regression model and residuals, is fulfilled according to \( p < 0.05 \), while the second test, also known as the LOF test, comparing the model and replicate errors with each other, is fulfilled according to \( p > 0.05 \) which is an important diagnostic test for model validity, but cannot be performed if replicates are missing [13, 37]. Alternatively, the significance of the regression model can be evaluated by using a critical \( F \)-value for the model and LOF from literature according to the \( F \)-table, in which the \( F \)-value and the corresponding DF of the model as well as the respective residual error term are summarized for a significance or critical reference value equal to the tested model ratio (normally, 95\%, thus, for \( p = 0.05 \)) [13, 22, 27]. Hence, regression models are accepted or statistically significant according to \( F \)-value of the model > corresponding critical \( F \)-value, and \( F \)-value of LOF < critical \( F \)-value. In other words, the \( F \)-test is generally providing a method for the determination of significant differences between different variation sources in the experimental results, i.e., the significance of regression (SOR), the lack-of-fit (LOF), and the coefficient of determination \( (R^2) \), at which the ANOVA table for response variables is including the mean squares of all variance sources (e.g., regression, LOF, etc.) and is finally comparing the relative to the residual (prediction error) variance [17, 19, 30, 38]. When full second-order models (models covering all two parameter interactions) are not accepted by the previous mentioned tests, they can be improved by removing model terms, until the obtained
conditions are fulfilled, at which the significance of each reduced model term is $F$-tested according to eq 3 [17, 45].

$$ F = \frac{SS_{\text{diff}} / (p-g)}{SS_{\text{re}} / (n-g)} $$

(3)

Here, $SS_{\text{re}}$ is referring to the sum of squares of the residuals in the reduced model, while $SS_{\text{diff}}$ is related to the difference between the sum of squares of residuals in the complete and reduced models, at which the complete model is containing all considered interaction terms [17, 19, 45]. Furthermore, the letters $n$, $p$ and $g$ are referring to the total number of experiments and the number of parameters in the complete and reduced model, respectively, at which the reduced model is only accepted, if the $F$-significance is lower than 95 % [17, 45]. In case, that no improvement is achieved by removing model terms, the model can be improved by the addition of third-order terms containing only excluded parameters with the highest significance, until the obtained conditions are fulfilled [11, 17, 19, 30, 45]. Thereby, third-order polynomial models are usually used for model fitting because first- and second-order models are generally suffering from LOF [36]. The significance of each added model term is evaluated by the $F$-test according to eq 3, at which the model is only accepted, if the $F$-significance is higher than 95 % [19]. Hence, models are accepted when the significance of SOR is higher than 95 %, while the one of LOF is lower than 95 % [45].

However, if one of these conditions is not fulfilled by a given model, the model is only accepted when $R^2 > 0.95$ meaning that more than 95 % of the variance of the data are "explained" by the model [17, 22, 28, 45]. Hence, additionally to the parameters in the $F$-test, ANOVA is including other statistically useful goodness-of-fit parameters for the evaluation of models [13, 65]. One parameter is the explained variation or coefficient of determination ($R^2$) mentioned above, which is lying between 0 and 1 and representing the classical coefficient or parameter for model evaluation [13]. Unfortunately, $R^2$ is very sensitive to the DF and it is possible to make $R^2$ arbitrarily close to 1 by the addition of more model terms or variables,
Regardless whether variables are significant or not, thus misleading the true behavior of the model due to a higher $R^2$ value, which is finally, resulting in a poor prediction of estimated response variables [13, 30, 33, 59]. In order to overcome or reduce this sensitivity and deficiency of $R^2$, the explained variance or adjusted coefficient of determination ($R^2_{adj}$) is introduced, thus creating a more useful goodness-of-fit-parameter, which is adjusted to the DF and can be used to verify the amount of the reduced response variability by using independent variables in the model [13, 17]. Furthermore, the value of $R^2_{adj}$ is usually lower than that of $R^2$, at which $R^2$ is normally decreased as a result of the model revision when less useful model terms are removed, whereas $R^2_{adj}$ will be generally not increased and should substantially remain unchanged with addition of variables [13, 17]. In fact, the value of $R^2_{adj}$ will be often decreased by addition of unnecessary model terms, at which the values of $R^2$ and $R^2_{adj}$ are ideally close to each other, indicating that all used model terms are necessary for a correct model building [17, 30, 59]. However, if too many insignificant terms are included in the model, there is a large difference between the values of $R^2$ and $R^2_{adj}$ [59, 66]. Moreover, the predicted variation or coefficient of predicted variation ($Q^2$), also called the goodness of prediction or cross validation, is often employed as a third parameter in the regression analysis for evaluating the predictive power of a model, and is therefore a primary attractive parameter in regression modelling [13, 19]. The values of $R^2$ and $R^2_{adj}$ are significantly or slightly decreased by removing less useful model terms, while the value of $Q^2$ is normally increased, even though irrelevant model terms are excluded from the model, thus confirming that a model revision was appropriate [13]. Hence, $Q^2$ is the most realistic parameter of the three regarding a more accurate model prediction, which is not directly provided by ANOVA, but can be derived from similar mathematical operations [13]. On the whole, the significance of a model can be evaluated by using ANOVA on the basis of multiple regression analysis, $F$-test and 95% confidence level [11, 17, 19, 30, 37].
2.5 Validation of model

The last DoE step is the validation of the previously analyzed mathematical model. Additional experiments are performed by using the optimal values of significant factors in order to validate a new model [7, 14]. In this context, an adequate validation of the model is confirmed by a good correlation or small difference between determined and predicted response values [7, 14, 17, 19]. Finally, the model can be used for an adequate prediction of target response variables under any set of variable combinations.

3. Applications of PEG–salt ATPS using DoE

All previously discussed DoE steps have been sequentially demonstrated by Lu et al. [16]. They have evaluated the recovery and purification of lysozyme from crude chicken egg white in a PEG 4000–potassium citrate system. Initially, they carried out screening experiments using a 2⁵−¹ fFD with three center-points in order to determine the significant factors in lysozyme partitioning. In this context, five independent variables (factors) covering PEG concentration (X₁, wt. %), potassium citrate concentration (X₂, wt. %), potassium chloride (X₃, wt. %), pH (X₄) and temperature (X₅, °C) were taken into consideration, while the purification factor (PF) and activity yield (Y₁, %) of lysozyme were set as the response variables (responses). Based on the experimental data obtained by this design, linear regression models/equations were established for each of the response variables (Y₁ and PF), at which the magnitude of the coefficients of these models/equations could be generally utilized for an evaluation of the contribution of the corresponding factors to the response variables [54]. According to the statistical significance test, the concentration of potassium citrate (X₂, wt. %) and potassium chloride (X₃, wt. %) were significant factors (p < 0.05) for PF, while the regression equations denoted that the pH (X₄) was a significant factor for both response variables. Based on the first-order model established by the factorial design, a new series of experiments was performed in the direction of steepest ascent in order to improve the
purification factor \((PF)\) and activity yield \((Y_1, \%)\) of lysozyme, and finally reach the vicinity optimum. Furthermore, the specific activity \((Y_2, \text{U/mg})\) of lysozyme was calculated, serving as a third response variable. Subsequently, the systems were further optimized by RSM using a CCF design, resulting in a second-order model with corresponding regression equations for predicting optimal response variables \((PF, Y_1 \text{ and } Y_2)\). In this context, high values of \(R^2\) and \(R^2_{adj}\) for all three response variables \((PF, Y_1 \text{ and } Y_2)\) were calculated, indicating a good degree of correlation between the actual and predicted data \([33; 67]\). Furthermore, a statistical analysis of the recovery and purification of lysozyme using the predicted second-order regression models/equations was evaluated by ANOVA considering the \(F\)-test or \(F\)- and \(p\)-values, as well as LOF which revealed that all regression models/equations were statistically significant at the 99.999 % confidence level (\(p\)-values < 0.05; \(p\)-value(s) > \(F\)-value(s) < 0.0001; \(p\)-value of LOF > 0.05), thus confirming an excellent adequacy of the quadratic models to the experimental data \([27, 68, 69]\). Additionally, an analysis of response surface plots revealed an optimized system composed of 18 wt. % PEG, 16 wt. % potassium citrate and 3.75 wt. % potassium chloride at pH 5.5 and 30 °C. Under those experimental conditions, lysozyme was recovered in the top phase in one single extraction stage containing a purification factor, activity yield and a specific activity of 21.11, 103 % and 31,100 U mg\(^{-1}\), respectively, which were validated by several parallel experiments performed at the factor levels determined by RSM/CCF design. Thereby, the observed and predicted results of these experiments had a strong similarity, thus indicating the suitability of RSM for an optimization of the recovery and purification of lysozyme in ATPS and the accuracy of the regression models for predicting the desired response variables \([10]\). Moreover, the feasibility of statistical modelling and model validation has been successfully evaluated and established for the optimization of different PEG–salt ATPS regarding partitioning, recovery and purification of various other proteins and enzymes by using DoE, as summarized in Tables 2–4.
According to these tables, usually a FFD/fFD is carried out in screening experiments for the determination of significant factors containing two to five factors (independent variables). Thereby, the most commonly used five independent variables are PEG MW ($X_1$), PEG concentration ($X_2$, wt. %), phase-forming salt concentration such as phosphate or citrate ($X_3$, wt. %), pH ($X_4$) and additional neutral salt like NaCl or KCl ($X_5$, wt. % or M), while the partition coefficient ($K$), product recovery/activity yield ($Y$, %) and purification factor ($PF$) are mostly set as the responses (dependent response variables). Furthermore, other independent variables, such as temperature (°C) and biomolecule/bioligand load/concentration (mg/mL or wt. %) and response variables, like the selectivity ($S$), are sometimes also taken under consideration. In general, the significant factors optimized in RSM are the concentrations of phase-forming components (PEG/phase-forming salt) and additional neutral salt, since these factors are most commonly optimized in RSM, while the temperature and concentration/load of biomolecule/bioligand tend to be generally not significant. Furthermore, the MW of PEG and the pH of system are sometimes also statistically significant and optimized in RSM. Thereby, the optimization of significant factors in RSM is usually performed by central composite designs (CCC/CCF). Furthermore, $K$, $Y$, $PF$ and the purity ($P$, %) are generally selected as response variables in RSM. Moreover, the ranges/values of the independent variables are specified in the different experimental designs in Tables 2–4 which are usually selected on the basis of the phase diagrams of ATPS according to the system parameters of the two aqueous phases. Furthermore, there is a specific, non predictable influence of the independent variables and corresponding interactions on each response variable in all PEG–salt ATPS or within the same aqueous system depending on the system parameters of the two liquid phases and the physicochemical/surface properties of the target biomolecules. Overall, the performance of the multivariate DoE is an important and necessary tool in order to provide an efficient
optimization and evaluation of all factors, interactions and responses in various PEG–salt ATPS due to the complex dependency of all system variables and biomolecule properties.

→ Tables 2–4

4. Conclusions

As evident from this review, the multivariate statistical DoE methodology has the feasibility to operate as a promising and efficient optimization technique for partitioning, recovery and purification of various proteins and enzymes in PEG–salt ATPS. Thereby, the process parameters or significant factors of these systems can be simultaneously changed and optimized by the DoE approach, allowing a fast, rigorous, efficient and cost-effective evaluation of the effects of different parameters or the significant factors on the selected response variables and their possible interactions, thus overcoming the restrictions of traditional optimization techniques, such as the OFAT method. Therefore, the DoE methodology has gained importance for a wide range of applications using PEG–salt ATPS in biotechnology and related fields, and hence this technique has emerged as a powerful tool for the efficient optimization of these systems. In this context, different DoE process steps containing various experimental designs, such as factorial and response surface designs, have been discussed and evaluated by statistical regression analysis as well as ANOVA. Finally, the statistical DoE approach has demonstrated to be highly suitable for the optimization of different PEG–salt ATPS, resulting in a significant reduction of the number of experiments and time, and consequently reduced overall cost, thus making the whole optimization process of these systems more cost-effective than traditional optimization techniques.

Full or fractional factorial designs (FFD/fFD) are predominantly carried out in screening experiments for determination of statistically significant factors, while these factors are commonly optimized in RSM by central composite designs (CCC/CCF). Furthermore, two to five factors (independent variables), such as PEG MW, PEG concentration, phase-forming
salt concentration, pH and additional neutral salt, are in general frequently investigated in DoE of PEG–salt ATPS, while $K, Y, PF, P$ and $S$ are commonly selected as the responses (dependent response variables). Since there is a complex dependency of all factors, responses, system parameters of the two aqueous phases and physicochemical/surface properties of the target biomolecules, the performance of the multivariate DoE approach is an important and necessary tool for an efficient optimization and evaluation of partitioning, recovery and purification of different biomolecules (e.g., proteins/enzymes) in PEG–salt ATPS. Overall, this review is providing all important aspects related to the optimization of PEG–salt ATPS covering different DoE process steps characterized by various experimental designs and the corresponding regression models/equations established by these designs which may further promote and facilitate the optimization of PEG–salt ATPS considering an evaluation of the partitioning behavior, recovery and purification of various proteins and enzymes in these systems.
References


[44] C.S. Silva, E. Bovarotti, M.I. Rodrigues, C.O. Hokka, M. Barboza, Evaluation of the effects of the parameters involved in the purification of clavulanic acid from...


Figure Captions

**Fig. 1** Schematic representation of a general process/system, adapted from [8, 9, 13].

**Fig. 2** General performance of a DoE process.

**Fig. 3** Illustration of a two-level factorial design matrix (a) covering three factors A, B, and C with their high (+) and low (−) levels corresponding to the geometrical representations of a $2^2$ (b) and $2^3$ (c) full factorial design (FFD) and $2^{3-1}$ (d) fractional factorial design (fFD).

**Fig. 4** Geometrical representations of two-level central composite designs (CCD's): central composite circumscribed (CCC) (a) and central composite face-centered (CCF) design (b) for three factors ($x_1, x_2, \text{ and } x_3$) and an overview of the experimental number in two-level CCD's covering 2–7 factors (c).

**Fig. 5** Illustration of a three-level Box–Behnken design (BBD) Matrix (a) covering three factors ($x_1, x_2, \text{ and } x_3$) and their levels (+1, 0, −1) for 15 experimental runs and the corresponding geometrical representation of the three-level BBD (b).
### Tables with Captions

#### Table 1: Statistical software packages used in DoE for optimization of PEG–salt ATPS.

<table>
<thead>
<tr>
<th>Statistical software packages</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Design-Expert® (Stat-Ease Inc., Minneapolis, MN, USA)</td>
<td>[25, 28, 33, 34, 36, 41, 51, 57, 59]</td>
</tr>
<tr>
<td>Minitab® (Minitab Inc., State College, PA, USA)</td>
<td>[6, 14, 46]</td>
</tr>
<tr>
<td>MODDE® (UMetrics AB, Umed, Sweden)</td>
<td>[17, 19, 37, 53]</td>
</tr>
<tr>
<td>SAS® (SAS Institute Inc., Cary, NC, USA)</td>
<td>[16, 18, 22–24, 32, 38, 43, 50, 52, 54–56]</td>
</tr>
<tr>
<td>SPSS® (SPSS Inc., Chicago, IL, USA)</td>
<td>[10, 48]</td>
</tr>
<tr>
<td>STATISTICA® (StatSoft Inc., Tulsa, OK, USA)</td>
<td>[16, 20, 28, 31, 34, 35, 42, 47, 52, 61, 65, 70–76]</td>
</tr>
</tbody>
</table>

#### Table 2: Several examples of PEG–salt ATPS using factorial experimental design.

<table>
<thead>
<tr>
<th>Design</th>
<th>ATPS</th>
<th>Protein</th>
<th>$X_1$</th>
<th>$X_2$</th>
<th>$X_3$</th>
<th>$K$</th>
<th>$Y$</th>
<th>$PF$</th>
<th>$S$</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2/FFD</td>
<td>PEG 8000–phosphate</td>
<td>α-Toxin</td>
<td>12.5-17.5</td>
<td>15-25</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>[20]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2/FFD</td>
<td>PEG 400–citrate</td>
<td>Lecitin ConGF</td>
<td>400-8000</td>
<td>20-24</td>
<td>15-20</td>
<td>6-8</td>
<td>X</td>
<td>X</td>
<td>[26]</td>
<td></td>
</tr>
<tr>
<td>2/FFD</td>
<td>PEG 1500–phosphate</td>
<td>Collagenase</td>
<td>1500-8000</td>
<td>12.5-17.5</td>
<td>10-15</td>
<td>6-8</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>[65]</td>
</tr>
<tr>
<td>2/FFD</td>
<td>PEG 8000–citrate</td>
<td>Phytase</td>
<td>400-8000</td>
<td>20-26</td>
<td>12-20</td>
<td>6-8</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>[73]</td>
</tr>
<tr>
<td>2/FFD</td>
<td>PEG 8000–citrate</td>
<td>Protease</td>
<td>400-8000</td>
<td>20-24</td>
<td>15-20</td>
<td>6-8</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>[74]</td>
</tr>
<tr>
<td>2/FFD</td>
<td>PEG 8000–citrate</td>
<td>Lecitin ConA</td>
<td>1500-8000</td>
<td>15-22</td>
<td>10-22</td>
<td>4-7</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>[72]</td>
</tr>
<tr>
<td>2/FFD</td>
<td>PEG 10000–citrate</td>
<td>Protease</td>
<td>150-20000</td>
<td>22-26</td>
<td>6-18</td>
<td>7-8-5</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>[75]</td>
</tr>
</tbody>
</table>

Factors (independent variables) used in factorial experimental designs: $X_1$: PEG MW; $X_2$: PEG concentration (wt. %); $X_3$: phase-forming salt concentration (wt. %); $Y$: product recovery/activity yield (%); $PF$: purification factor; $S$: selectivity.

#### Table 3: Several examples of PEG–salt ATPS using RSM.

<table>
<thead>
<tr>
<th>Design</th>
<th>ATPS</th>
<th>Protein</th>
<th>$X_1$</th>
<th>$X_2$</th>
<th>$X_3$</th>
<th>$K$</th>
<th>$Y$</th>
<th>$PF$</th>
<th>$P$</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2/CCC</td>
<td>PEG 1500–phosphate</td>
<td>mAb 2G12</td>
<td>12.5-17.5</td>
<td>15-25</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>[6]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2/CCC</td>
<td>PEG 3350–citrate</td>
<td>α-Amylase</td>
<td>11-17</td>
<td>12-20</td>
<td>2-10</td>
<td>X</td>
<td>X</td>
<td>[32]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2/CCC</td>
<td>PEG 600–phosphate</td>
<td>Lecitin ConBr</td>
<td>16.5-18.5</td>
<td>14-16</td>
<td>6.5-8.5</td>
<td>3.5-5.5</td>
<td>X</td>
<td>X</td>
<td>[43]</td>
<td></td>
</tr>
<tr>
<td>2/CCC</td>
<td>PEG 1500–citrate</td>
<td>BSA</td>
<td>17-21</td>
<td>18-20</td>
<td>6-8</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>[52]</td>
<td></td>
</tr>
<tr>
<td>2/CF</td>
<td>PEG 4000–phosphate</td>
<td>mAb/HCP</td>
<td>8-12</td>
<td>6-8</td>
<td>0-7</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>[53]</td>
<td></td>
</tr>
<tr>
<td>2/CF</td>
<td>PEG 3350–phosphate</td>
<td>IgG</td>
<td>12-14</td>
<td>10-12</td>
<td>0-15</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>[22]</td>
<td></td>
</tr>
<tr>
<td>2/CF</td>
<td>PEG 600–phosphate</td>
<td>IgG</td>
<td>8-12</td>
<td>10-12</td>
<td>6-8</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>[56]</td>
<td></td>
</tr>
<tr>
<td>2/2/2 CCD</td>
<td>PEG 1500–phosphate</td>
<td>Keratinase</td>
<td>1-17</td>
<td>21-25</td>
<td>0-8</td>
<td>X</td>
<td>X</td>
<td>[49]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BBD</td>
<td>PEG 4000–phosphate</td>
<td>Xylanase</td>
<td>4000-8000</td>
<td>7.5-11.25</td>
<td>15-22.5</td>
<td>X</td>
<td>X</td>
<td>[59]</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Factors (independent variables) used in RSM: $X_1$: PEG MW; $X_2$: PEG concentration (wt. %); $X_3$: phase-forming salt concentration (wt. %); $X_4$: pH; $X_5$: NaCl concentration (wt. %); $Y$: product recovery/activity yield (%); $PF$: purification factor; $P$: purity (%).

#### Table 4: Several examples of PEG–salt ATPS optimized by DoE.

<table>
<thead>
<tr>
<th>Design</th>
<th>ATPS</th>
<th>Protein</th>
<th>$X_1$</th>
<th>$X_2$</th>
<th>$X_3$</th>
<th>$K$</th>
<th>$Y$</th>
<th>$PF$</th>
<th>$S$</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2/FDF</td>
<td>PEG 4000–sulfate</td>
<td>Nisin</td>
<td>20.4-26</td>
<td>9-14.6</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>[23]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2/FDF</td>
<td>PEG 3400–phosphate</td>
<td>GUS</td>
<td>14-8-2</td>
<td>4-8</td>
<td>6-9</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>[10]</td>
<td></td>
</tr>
<tr>
<td>2/FDF</td>
<td>PEG 4000–citrate</td>
<td>Lysozyme</td>
<td>15-25</td>
<td>15-20</td>
<td>6-9</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>[16]</td>
<td></td>
</tr>
<tr>
<td>2/FDF</td>
<td>PEG 3350–citrate</td>
<td>mAb/HCP</td>
<td>11-17</td>
<td>5-5.5</td>
<td>7.2</td>
<td>8-15</td>
<td>X</td>
<td>X</td>
<td>[25]</td>
<td></td>
</tr>
<tr>
<td>2/FDF</td>
<td>PEG 3000–sulfate</td>
<td>Thaumatin</td>
<td>3000/1000</td>
<td>3000/1000</td>
<td>10-15</td>
<td>13/18</td>
<td>8-18</td>
<td>0.1/1.1</td>
<td>[33]</td>
<td></td>
</tr>
<tr>
<td>2/FDF</td>
<td>PEG 3400–sulfate</td>
<td>Lysozyme</td>
<td>3400/8000</td>
<td>15-10</td>
<td>13/18</td>
<td>6/8</td>
<td>0.1/1.2</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>2/FDF</td>
<td>PEG 8000–citrate</td>
<td>α-Amylase</td>
<td>600/8000</td>
<td>10-16</td>
<td>8/12</td>
<td>6/7</td>
<td>0.2/1.6</td>
<td>X</td>
<td>X</td>
<td>[10]</td>
</tr>
</tbody>
</table>

Factors (independent variables) used in experimental designs: $X_1$: PEG MW; $X_2$: PEG concentration (wt. %); $X_3$: phase-forming salt concentration (wt. %); $X_4$: pH; $X_5$: NaCl (KCl in Ref. 10, 16, concentration (wt. %) or concentration (M) in [33, 46, 50]; responses (dependent response variables) used in experimental designs: $K$: partition coefficient; $Y$: product recovery/activity yield (%); $PF$: purification factor; $S$: selectivity. Further factors (independent variables) used in screening designs: [10]: PBD: temperature: 2/20 °C; citrate/phosphate; NaCl/KCl; biogeland starch/sucrose; biogel and sarcosine; 0-0.5 wt. % [16]; 2°FDF: temperature: 25-45 °C; [25]; 2°FDF: biocatalyst load: 1.5 mg/mL; [33]; 2°FDF also screening of PEG–salt ATPS: 13/17 wt. % phosphate and 10/14 wt. % PEG, while all other factors were the same like in the PEG–sulfate systems [55]; 2°FDF: temperature: 20–30 °C.
Fig. 1
Fig. 2
Fig. 3

<table>
<thead>
<tr>
<th>Run</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

2-level factorial Design Matrix

2² FFD

2³ FFD

2³-1 FFD
Fig. 4

<table>
<thead>
<tr>
<th>Factors</th>
<th>Experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>8 + 3</td>
</tr>
<tr>
<td>3</td>
<td>14 + 3</td>
</tr>
<tr>
<td>4</td>
<td>24 + 3</td>
</tr>
<tr>
<td>5</td>
<td>26 + 3</td>
</tr>
<tr>
<td>6</td>
<td>44 + 3</td>
</tr>
<tr>
<td>7</td>
<td>78 + 3</td>
</tr>
</tbody>
</table>

Overview of CCD’s with 2–7 Factors
Fig. 5

<table>
<thead>
<tr>
<th>Run</th>
<th>$x_1$</th>
<th>$x_2$</th>
<th>$x_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-1</td>
<td>-1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>-1</td>
<td>+1</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>+1</td>
<td>-1</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>+1</td>
<td>+1</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>-1</td>
<td>0</td>
<td>-1</td>
</tr>
<tr>
<td>6</td>
<td>-1</td>
<td>0</td>
<td>+1</td>
</tr>
<tr>
<td>7</td>
<td>+1</td>
<td>0</td>
<td>-1</td>
</tr>
<tr>
<td>8</td>
<td>+1</td>
<td>0</td>
<td>+1</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>-1</td>
<td>-1</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>-1</td>
<td>+1</td>
</tr>
<tr>
<td>11</td>
<td>0</td>
<td>+1</td>
<td>-1</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td>+1</td>
<td>+1</td>
</tr>
<tr>
<td>13</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

3-level Box–Behnken Design Matrix
Highlights

- Characterization of a DoE process for optimization of ATPS by different steps.
- Favoring screening of significant factors in ATPS using factorial designs (FFD/fFD).
- Favoring optimization of ATPS by RSM using central composite designs (CCC/CCF).
- Evaluation of experimental designs by statistical regression analysis and ANOVA.
- Summary and evaluation of several statistical software packages used in ATPS.