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Method development and validation for the chiral separation of peptides in the presence of cyclodextrins using capillary electrophoresis and experimental design

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Abstract

The present study describes the application of statistical experimental design to the optimization of enantioselective separations of peptides in capillary electrophoresis in order to obtain optimal operating conditions for routine work. Hydroxypropyl-β-cyclodextrin was used as chiral selector and Ala–PheOMe as model peptide. The experiments were performed according to a face centered cube response surface experimental design for obtaining information how the factors such as concentration of the chiral selector, pH, buffer concentration and voltage affected the two response goals, resolution and analysis time. In order to achieve the simultaneous optimization of these two major electrophoretic performance goals for efficient and fast separation, the Derringer desirability functions were tested. While in the predefined experiments the analysis time for baseline separation was 25 min the desirability functions proposed a CE method, which diminished the analysis time and permitted the complete separation of the peptide enantiomers within 9 min.

Keywords: Enantiomer separation; Method development; Experimental design; Cyclodextrins; Peptides

1. Introduction

Analytical enantioresolution of racemic substances is of great importance since enantiomers often present different pharmacokinetic, pharmacological or toxicological properties. The US Food and Drug Administration (FDA) defines as a prerequisite for developing new stereoisomeric drugs, the known stereoisomeric composition of the new drugs as well as the development of a validated and robust analytical technique for the quantification of these isomers [1]. In recent years there has been an increasing interest in biologically active peptides and peptidomimetics as pharmaceutical drugs. As a highly efficient separation technique capillary electrophoresis (CE) has also been used for the analysis of peptide enantiomers [2,3].

Cyclodextrins are very well known chiral selectors in chromatography [4] and the most widely used selectors in CE [5]. In contrast to common chiral phases, the chiral selectivity is given by a combina-

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tion of the inclusion process and the stereogenic centers of the cyclodextrin molecule. Each of the glucose units of which the cyclodextrin is formed contains five chiral centers and two secondary hydroxyl groups at the wider rim of the cavity that enable hydrogen bonding. If one of two isomers fulfils the three-point interaction with the cyclodextrin cavity, as the Dalgliesh theory supports, then the separation of stereoisomers is highly efficient. In this paper, the development of a methodology for the chiral separation of enantiomeric dipeptides by CE using hydroxypropyl-β-cyclodextrin (HPβCD) as chiral selector and Ala–PheOMe as model peptide is proposed.

Peptides are of general pharmaceutical interest as many newly developed drugs are either peptides or peptidomimetics. Unlike most non-peptide drugs, peptides and amino acid-derived structures are amphoteric compounds which have been shown to exhibit unexpected behavior in CE such as reversal of the enantiomer migration order upon changing the concentration of the chiral selector [6] or the pH of the background electrolyte [7–9]. Therefore, it was of interest to see if experimental design may also be applied to optimizing a CE separation of peptides.

The selection of the optimization criteria in the field of CE can be any of the typical terms such as the apparent separation factor α ($\alpha = t_2/t_1$), resolution between adjacent peaks, migration time etc. Although in most cases in experimental design one criterion is optimized at a time [10,11], it is usually necessary to take several of them into account. When, for instance, one intends to optimize only the resolution of two peaks in CE, the expression for resolution between the two peaks is quite straightforward. The problem becomes more complicated if an analytical chemist wants to optimize more than one CE criterion and wishes to have at the same time a good (baseline) separation between analyzed species and to perform many analyses in a short time. This requires a criterion, which combines two different terms (resolution R and migration time) and allows their simultaneous optimization.

A combined electrophoretic criterion for the simultaneous optimization, usually called a chromatographic response function (CRF) has been used in the literature in mathematical expressions, which consisted of a factor related to the migration time and a factor related to the separation quality [12–15]. While such CRF expressions could range from simple to complicated ones, it is sometimes quite difficult for the analyst to derive appropriate and simple CRF functions in order to optimize the CE conditions.

A different approach to the simultaneous optimization of different criteria in CE could be the application of Derringer's desirability function D [16–18], which is based on the transformation of the measured property to a dimensionless scale d for each criterion. The scale of the desirability function ranges between $d=0$ for a completely undesired response, to $d=1$ for a fully desired response, above which further improvements would have no importance. Next, the overall quality D is calculated combining the desirability values obtained for the different criteria by applying the geometric mean: $D=(d_1 \times d_2 \times \ldots\ d_m)^{1/m}$. It is possible also to attribute importance masses to individual d_i functions and then the total D function has the form of $D=(d_1^{w1} \times d_2^{w2} \times \ldots \times d_m^{wm})^{1/(w1+w2+\ldots+wm)}$. An algorithm is then applied to the D function in order to determine the set of variable values that maximize it. When the different criteria are globally optimal D reaches its highest value. A mathematical representation of desirability functions applied to response variables Y_i is as follows:

$$d_i = 0 \quad \text{if} \quad Y_i \le Y^-$$

$$d_i = \left(\frac{Y_i - Y^-}{Y^+ - Y^-}\right)^r \quad \text{if} \quad Y^- \le Y_i \le Y^+$$

$$d_i = 1 \quad \text{if} \quad Y_i \ge Y^+$$

In this type of transformation the user has the free choice to select the minimum value of criterion Y_i^-, the value of the criterion Y_i^+ beyond increasing response yields no further benefit and the parameter r (which is in fact a kind of weighing factor), selection of which favors the values near Y_i^- ($r<1$) or Y_i^+ ($r>1$).

Although many studies in the literature present the separation optimization of various substances using capillary electrophoresis and experimental design [19–22], only a few use desirability functions for the multiresponse optimization. These include the separation of epinephrine enantiomers [23], sodium dodecyl sulfate and sodium cholate [24] and the

separation of rare-earth–metal ions [25]. The aim of this study was the simultaneous optimization of the analysis time and the resolution of enantiomeric peptides in CE using cyclodextrins as chiral selector in order to set up a routine method for the analysis of similar samples. In the present study, the application of the contour profiler (see later) as implemented in the software JMP DISCOVERY version 4 (SAS Institute SAS Campus Drive, Cary, NC, USA) that brings up an interactive contour profiling facility is implemented for first time in the literature of CE separations. This is useful for optimizing response surfaces graphically as well as indicating more than one set of optimum conditions for the current problem of the baseline separation of peptides using CE.

The experiments for the optimization were performed according to the face centered cube (FCC) [26,27] a special kind of response surface experimental design, which are the best designs for the purpose of modeling and optimization. The influence of the four different factors (pH, buffer concentration, chiral selector concentration and voltage) on the quality of the electropherograms was evaluated. The experiments performed under the conditions proposed by the FCC design in randomized order (to minimize random effects) and the responses of interest (migration time of the second peak and the resolution R) were recorded for further analysis.

2. Materials and methods

2.1. General

L-PheOMe, D-PheOMe, *Z*-L-Ala *N*-hydroxysuccinimide ester and *N*-*Z*-D-Ala *N*-hydroxysuccinimide ester were obtained from Bachem (Heidelberg, Germany). (2-hydroxypropyl)-β-cyclodextrin (HPβCD, degree of substitution 0.6) was from Fluka (Deisenhofen, Germany). L-Ala–L-PheOMe and D-Ala–D-PheOMe were prepared by reaction of protected amino acid hydroxysuccinimide ester with L-PheOMe and or D-PheOMe, respectively, in dimethylformamide [28] followed by hydrogenolytic deprotection. All other chemicals were of analytical grade. Buffers and sample solutions were prepared in double-distilled, deionized water, filtered (0.47 μm), and degassed by sonication.

2.2. Capillary electrophoresis

All experiments were performed on a Beckman P/ACE 5510 instrument (Beckman Coulter, Unterschleissheim, Germany) equipped with a diode array detector at 20°C using 50 μm I.D., 360 μm O.D. fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA). The effective length of the capillaries was 40 cm, the total length was 47 cm. UV detection at 215 nm was performed at the cathodic end. Sample solutions (100 μg/ml peptide dissolved in water) were introduced at a pressure of 3447.4 Pa for 3 s (0.5 p.s.i.). All separations were performed in sodium phosphate buffers. The pH was adjusted using 100 m*M* phosphoric acid after the addition of the HPβCD. Between the analyses the capillaries were washed 1 min with 100 m*M* phosphoric acid and 3 min with the run buffer.

3. Results and discussion

3.1. Choice of the factors

The CE system was optimized in order to obtain satisfactory overall resolution in a short analysis time. The effectiveness of cyclodextrins as chiral selectors in CE systems, including the separation of peptide enantiomers, is well documented in literature [2,3,5]. The concentration of HPβCD in the buffer is an important factor to be optimized. In addition, the buffer pH is one of the most important factors in CE as it may affect the charge of the analytes and the chiral selector (and by this the binding characteristics between analyte and the selector) as well as the electroosmotic flow (EOF). The pH range of 2.5–4.0 evaluated in the present study was based on previous experience with separations of peptide enantiomers [9]. Within this pH range the dipeptide ester used is protonated as the pK_a of the amino group is about 9. Moreover, the EOF is not predominant in the selected pH range. Buffer concentration and the applied voltage were other factors that were considered. Temperature was not included.

The appropriate selection of the experimental

Table 1
Factors and experimental domain during the face central composite design

Factor	Low level	High level
pH	2.5	4
Buffer	10	100
Voltage (kV)	10	25
CD concentration (M)	10	100

domain for each of the four factors for the enantioseparation of peptides was made from prior experience and knowledge of the assay system (Table 1). The considered responses were the resolution, R (to approach a defined target value -1.5 in the present study) and the analysis time, t_2 (the migration time of the second migrating peak, which was attempted to be minimized).

3.2. Experimental design — statistical analysis

3.2.1. ANOVA tables

The experiments used for modeling and optimization were performed on three levels for each of the four factors using the face centered cube response surface experimental design. The experimental matrix consisted of twenty-four runs of different combinations of factor levels plus eight experiments at the central level of each factor, performed on 2 consecutive days in order to test the inter-day experimental variability. The estimate of the experimental error variance allowed the significance of the coefficients to be evaluated and the analysis of variance (ANOVA) to be carried out (Table 2). The ANOVA showed that the regression models assumed were significant, indicating that the change in the observed responses was due to the level change of the examined factors. In both cases the R^2 values indicated that the variation in the responses around the mean could be attributed to the examined factors rather than to random error. In the ANOVA tables it should be noted the 17 degrees of freedom (df) for the error term (error df) which will be used for the lack of fit table (see later). The error df is the total df (31 in the present case) minus the df for the model (14, since there are 15 fitting parameters, 14 derived from the four factors — main, quadratic and two-way interactions — plus the intercept). The inter-day variability was insignificant as resulted from the one-way ANOVA for the repeated measurements in 2 consecutive days ($P=0.9$). Furthermore the assumption for homogeneity was fulfilled since the Levene's test of homogeneity ($P=0.4$) indicates that the variances are homogeneous. Moreover, by examining the lack of fit diagnostic test (the difference between the residual error from the model and the pure error) we could conclude that an insignificant proportion of error is explained by lack of fit ($P=0.71$). Finally, the examination of the residuals showed that there is not any obvious pattern, they are structureless and therefore the model can be considered adequate.

3.2.2. Parameter estimates

In order to identify the active factors, upon whose alteration a statistically significant variation of the response was observed, the analysis of coefficients was considered. Table 3 shows the estimates (coefficients of the linear model found by least squares) of the parameters in the linear model and a t test for the hypothesis that each parameter is zero. The statistically significant coefficients were those that their absolute value was greater than zero with a probability of 95%. The confidence interval was calculated starting from the estimate of coefficients,

Table 2
ANOVA tables and summary of fit for each of the two responses (t_2 and R)

Source	df	Sum of squares	Mean square	F ratio
Response t_2 (R^2 0.998)				
Model	14	4970.9765	355.070	852.0525
Error	17	7.0843	0.417	Prob.$>F$
C. total	31	4978.0608		<0.0001
Response resolution (R^2 0.936)				
Model	14	7.8236393	0.558831	15.2341
Error	17	0.6236104	0.036683	Prob.$>F$
C. total	31	8.4472497		<0.0001

Table 3
Estimates (coefficients of the linear model found by least squares) of the parameters in the linear model and hypothesis test t

Term	Response t_2				Response resolution			
	Estimate	S.E.	t Ratio	Prob.$>\|t\|$	Estimate	S.E.	t Ratio	Prob.$>\|t\|$
Intercept	21.098389	0.441453	47.79	<0.0001	1.3310909	0.130976	10.16	<0.0001
pH	−2.618213	0.215247	−12.16	<0.0001	0.010517	0.063863	0.16	0.8711
Buffer	2.9410202	0.174126	16.89	<0.0001	0.5129744	0.051662	9.93	<0.0001
Voltage	−11.95004	0.174217	−68.59	<0.0001	−0.016133	0.051689	−0.31	0.7588
CD	8.0101903	0.174126	46.00	<0.0001	−0.051374	0.051662	−0.99	0.3340
pH×buffer	0.8935213	0.233056	3.83	0.0013	0.0594973	0.069146	0.86	0.4015
pH×voltage	0.4562222	0.233354	1.96	0.0672	0.1071314	0.069235	1.55	0.1402
Buffer×voltage	−1.125461	0.161335	−6.98	<0.0001	0.0838135	0.047867	1.75	0.0980
pH×CD	−1.723051	0.233056	−7.39	<0.0001	0.0677443	0.069146	0.98	0.3410
Buffer×CD	1.2181101	0.161284	7.55	<0.0001	0.0855691	0.047852	1.79	0.0916
Voltage×CD	−4.505461	0.161335	−27.93	<0.0001	0.0537684	0.047867	1.12	0.2769
pH×pH	−0.591179	0.30069	−1.97	0.0658	0.1813806	0.089213	2.03	0.0580
Buffer×buffer	−1.016023	0.406714	−2.50	0.0230	−0.094794	0.12067	−0.79	0.4429
Voltage×voltage	4.8375418	0.400741	12.07	<0.0001	−0.483468	0.118897	−4.07	0.0008
CD×CD	−1.048941	0.406714	−2.58	0.0195	−0.003053	0.12067	−0.03	0.9801

the estimate of standard error for each coefficient b_i and the value of t statistic for a 95% probability. The Prob.$>|t|$ is the probability of getting an even greater t statistics (in absolute value), given the hypothesis that the parameter is zero. From Table 3 it becomes evident which of the coefficients should be considered significant ($P>0.05$), marginally significant ($P\sim0.05$) and insignificant ($P<0.05$).

3.2.3. Normal plot

The normal probability plot of the ANOVA parameter estimates (Fig. 1) for the current model plotting the normal probabilities of the rank-ordered parameters on the y axis and the actual parameter estimates (optionally standardized) on the x axis, helps to distinguish between random noise and significant effects, which will be recognized as outliers. From Fig. 1 it is evident that for the response R the linear effect of the factor buffer (denoted as buffer (L) in the figure) and the quadratic effect of voltage (denoted as voltage (Q) in the figure) seem to be the most significant effects, in opposite directions, since they appear as distinct outliers compared to other effects which seem to be in the same straight line. Consequently, for the response t_2 the linear effects of the factors CD concentration and voltage and their two-way interaction (denoted as 3L×4L) seem to be the most significant effects. Similar conclusions are drawn from Bayes plot (Fig. 2).

3.3. Chromatographic response function

Generally, an electropherogram is mainly described by two parameters: (a) the migration time t, which characterizes the analysis time and it is considered as the migration time of the last analyte of the sample and (b) the resolution $R_S^{n,n+1}$ between two consecutive peaks n and $n+1$ for N analytes ($n=1,\ldots,N$) which is calculated as:

$$R_S^{n,n+1}=\frac{t^{n+1}-t^n}{(w^{n+1}+w^n)/2}$$

where t and w denote the migration times and the corresponding peak widths of two consecutive peaks n and $n+1$. In the case of coelution of two analytes (equal t) the resolution becomes zero, whereas in the case of total separation resolution $R_S\geq1.5$ is obtained. Excessive increase of the resolution should be avoided, since higher resolutions resulting in increasing analysis time are of no further benefit. Thus, in the present study, the desirability value $d=1$ was assigned the value 1.5 for R_S while desirability $d=0$ was assigned the values 1.4 and 1.9 for R_S (Fig. 3).

If the best electropherogram among those, which exhibit good enantioseparation, has to be chosen, one should pick the one with the shortest analysis time (t_2 in the present study). Therefore, the evaluation of the desirability of the analysis time is needed as well. The desirability value g of the electropherogram with

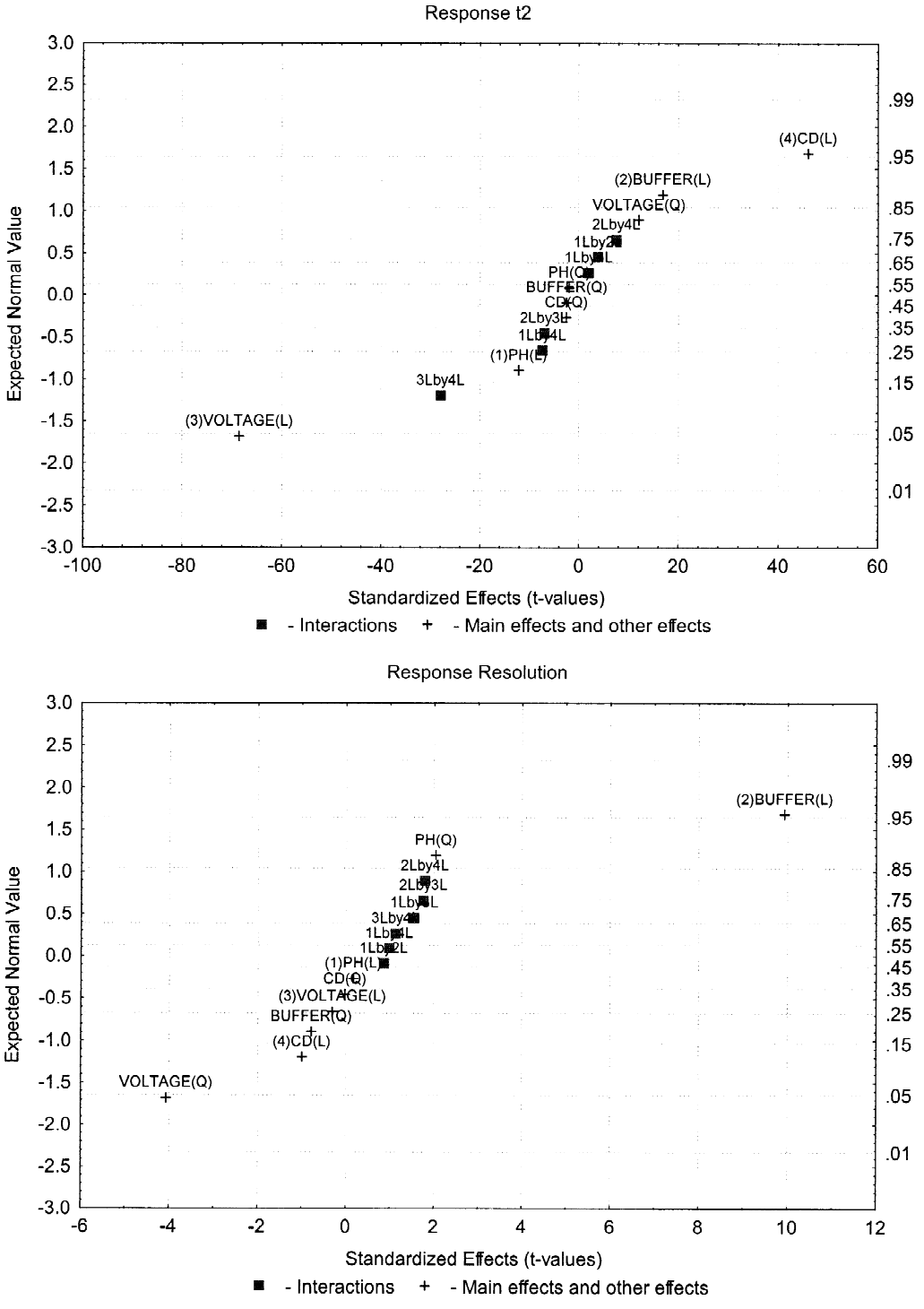

Fig. 1. Normal probability plots of the ANOVA parameter estimates: *y* axis represents the normal probabilities of the rank-ordered parameters and *x* axis represents the actual parameter estimates (optionally standardized).

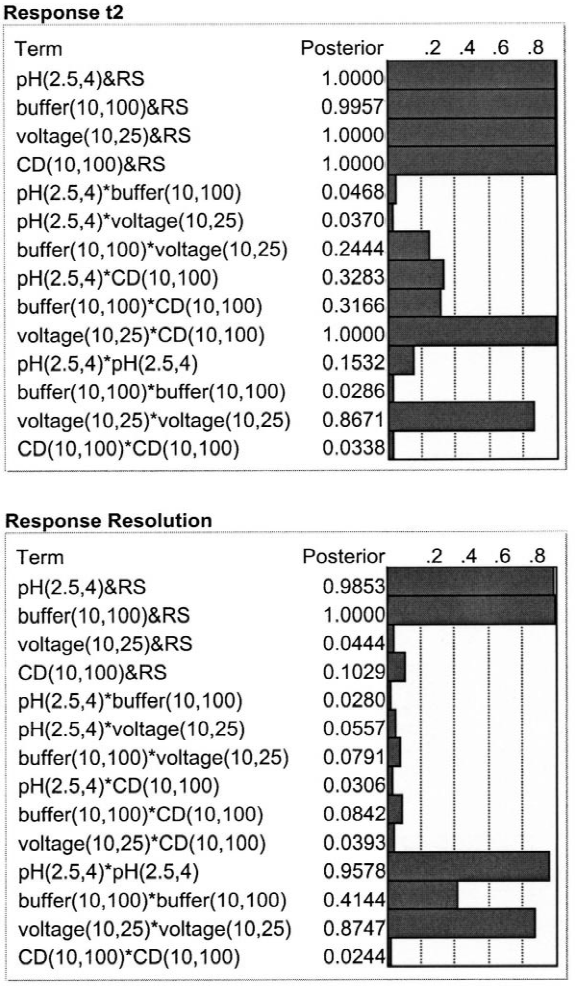

Fig. 2. Bayes plots and the posterior probabilities for the examined effects for both responses.

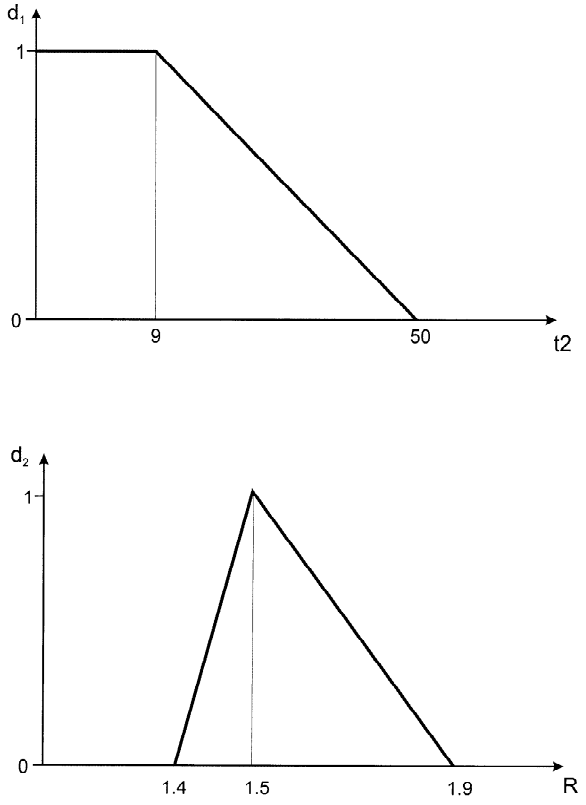

Fig. 3. Transformation of the responses t_2 and R into desirability values d. The fully desired and the undesired responses are reported.

regard to analysis time was obtained by defining the smallest analysis time as the most desirable (Fig. 3).

Finally, the chromatographic response function, CRF (i.e. the desirability of the overall appearance of an electropherogram in regard to the resolution R between the two isomers and the analysis time t_2), was calculated by multiplying the two desirability values f and g:

$$\mathrm{CRF}(f, g) = f \times g$$

Fig. 4 summarizes the results of the desirability function for both responses (R and t_2). In Fig. 4a the effect information appears for both responses, followed by the prediction profiler. The default profiles have all x variables set at their midpoint factor values. This last row of plots shows both the current desirability and the trace of desirabilities that result from changing one factor at a time. Fig. 4a displays the values for the resolution R_S (1.313) and the migration time t_2 (20.198 min), which correspond to the midpoint factor values and the corresponding desirability (0.779). Following, we define a 20 grid points for each of the four factors (resulting in $21\times21\times21\times21\times2=388\,962$ predicted values and 194 481 overall desirability scores). To contact the search for maximizing desirability, a general optimization method (simplex) is used. In Fig. 4b the values for the examined factors are shown, for which the desirability it is maximized (0.955) and correspond to an optimized electropherogram in which the

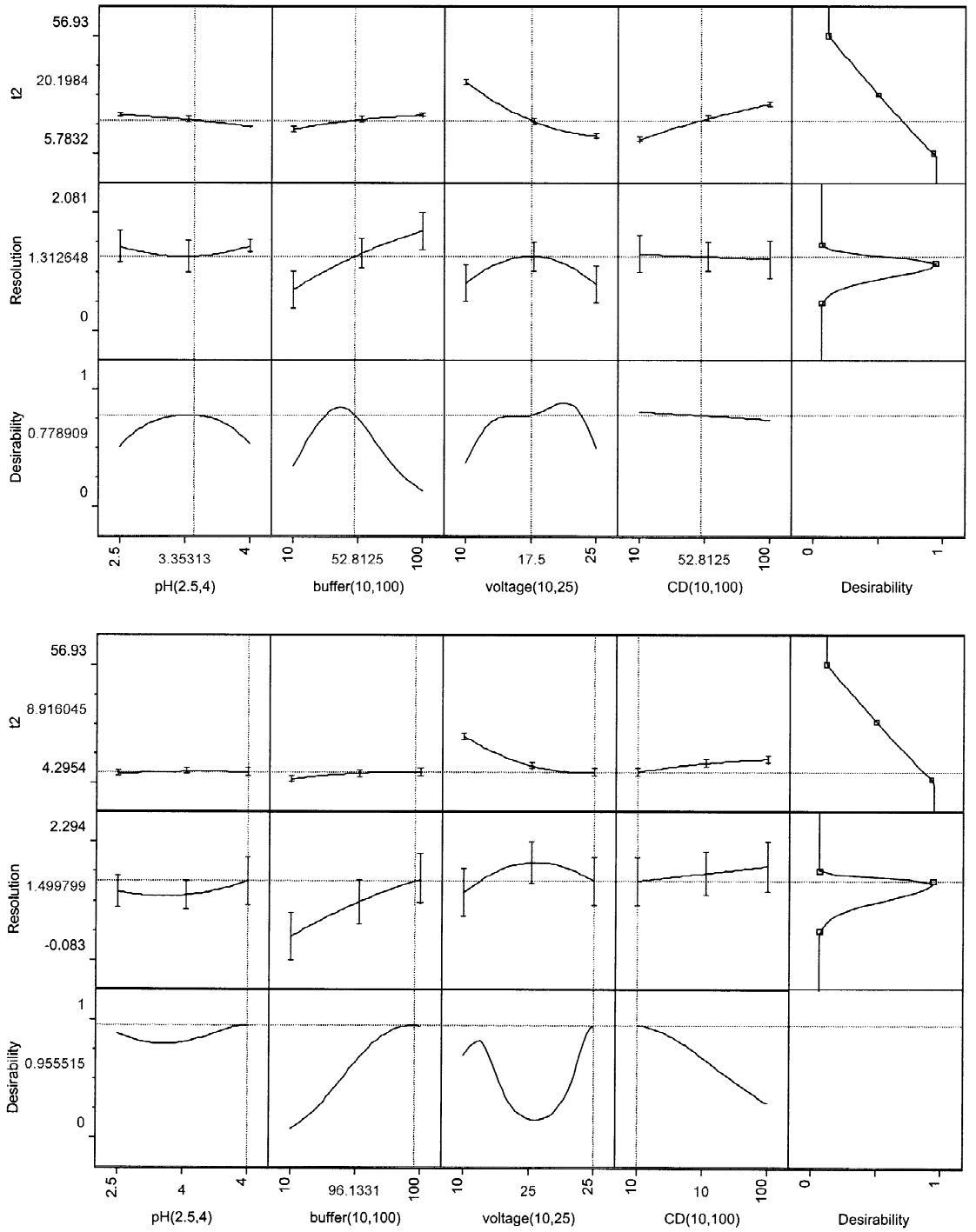

Fig. 4. Factor values and the corresponding responses before and after maximizing the overall desirability *D*. The numerical value beside the word desirability on the vertical axis is the geometric mean of the desirability measures. The maximum desirability value of 0.955 was achieved for $t_2 = 8.9$ min and $R = 1.5$.

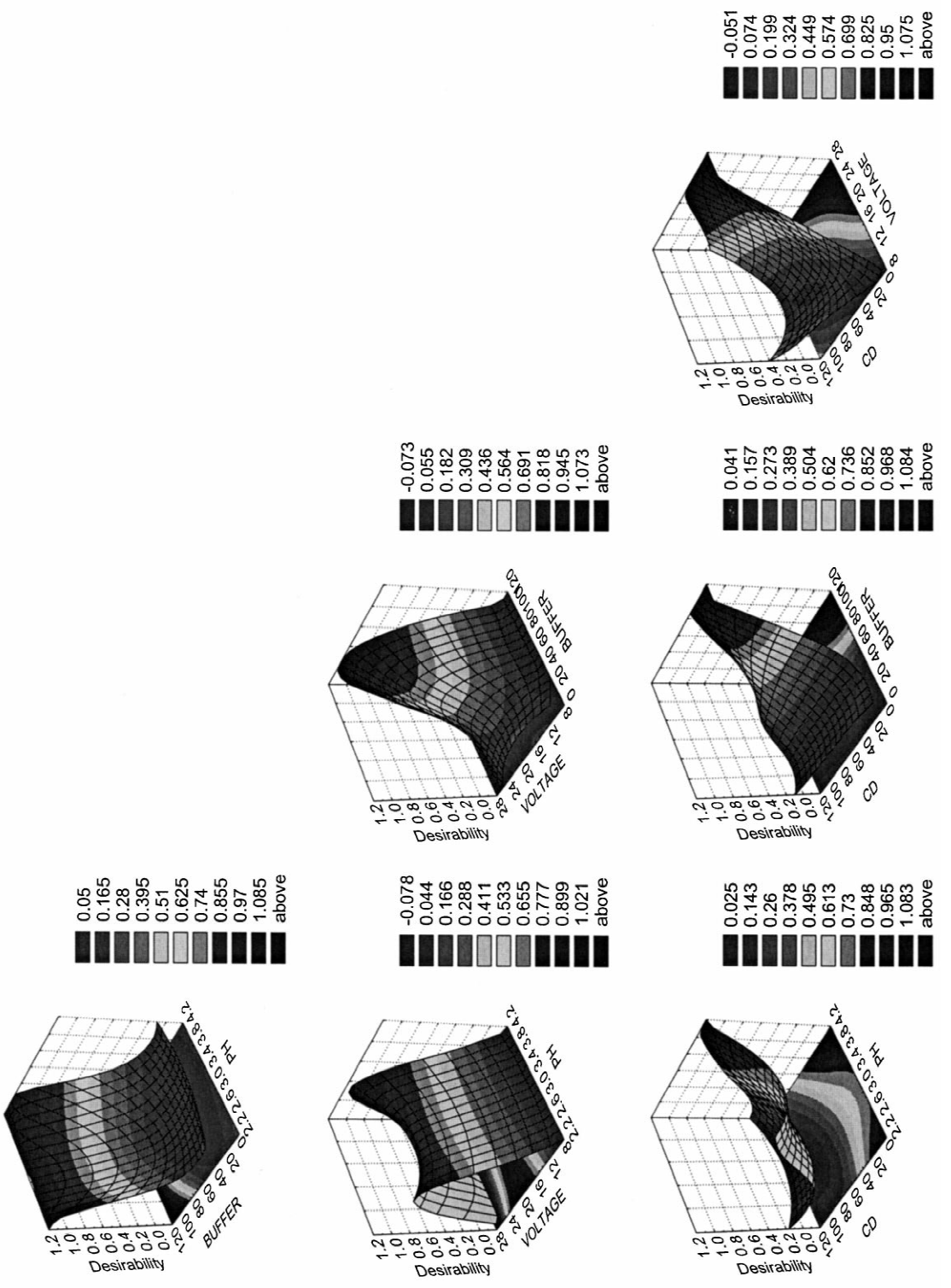

Fig. 5. Graphical representation of the overall desirability D for each group of two factors maintaining the other two factors at constant values.

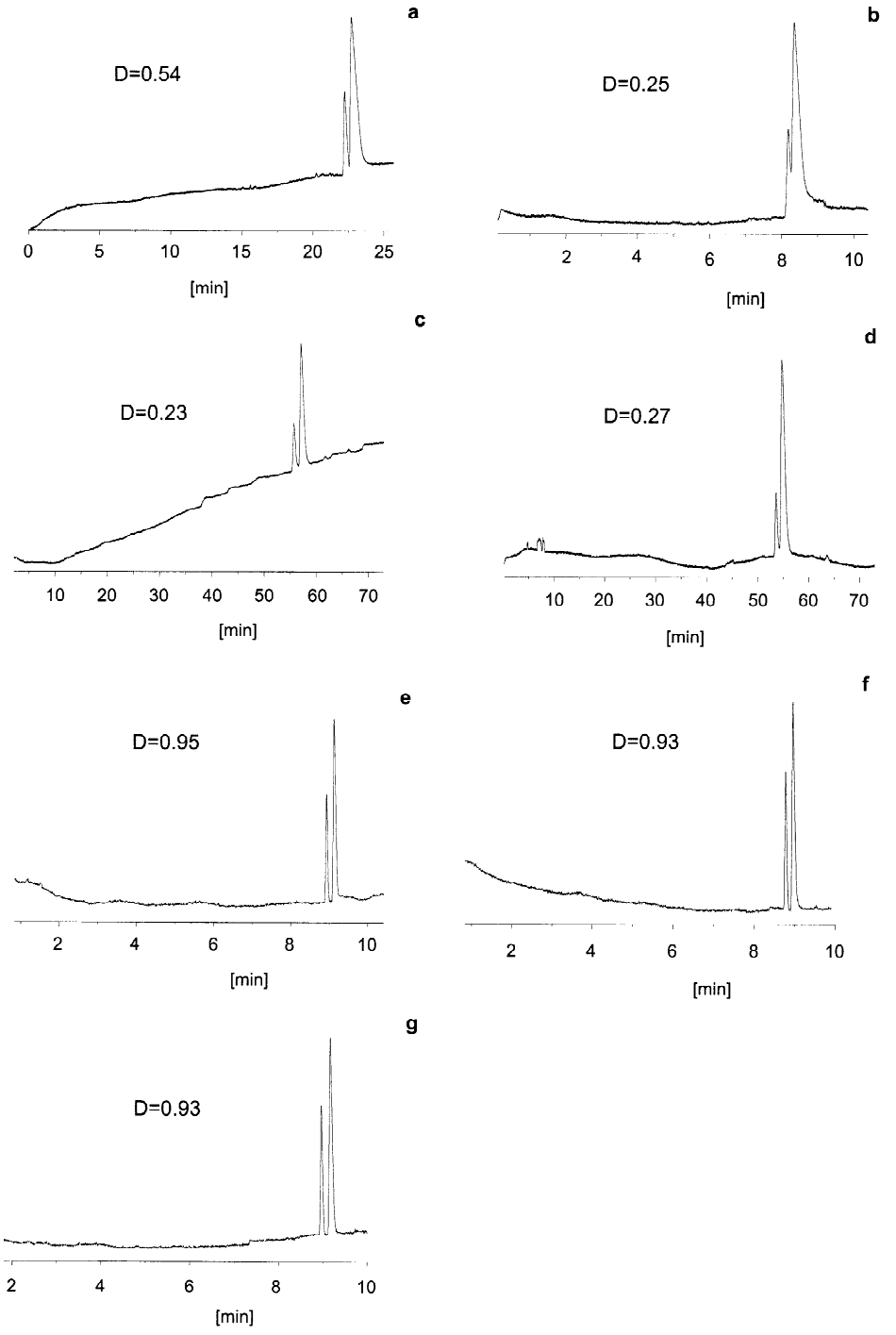

Fig. 6. Seven electropherograms at different conditions and their resulting D values. The higher D value corresponds to the electropherogram with the lower retention time and complete (baseline) separation ($R=1.5$). For a–g definitions see the text.

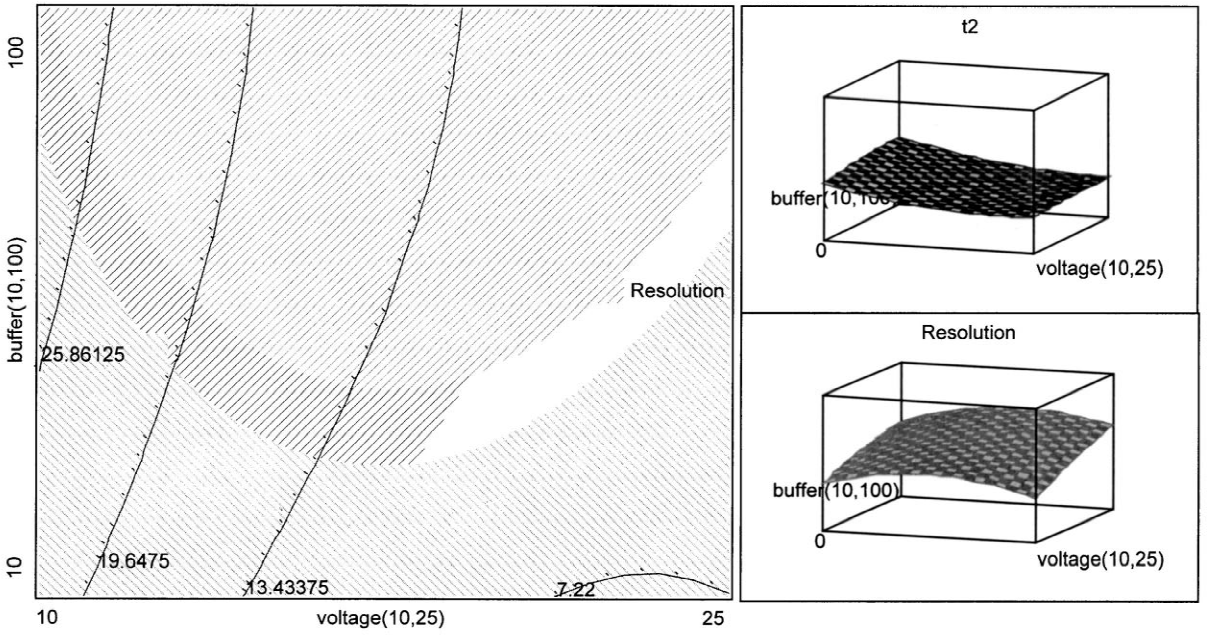

Fig. 7. White region represents the acceptable designs resulting from two overlapping contours.

resolution R approaches the target value of 1.5 and the analysis time t_2 is minimized (9 min). In Fig. 5 the fitting of the desirability values for each pair of the examined independent values (factors) is presented in 3-D surface plots, which was performed using the Spline method. The recommended experimental values for the examined factors, which appear in the horizontal axis of Fig. 4b were applied and the resulting electropherograms are shown in Fig. 6, which summarizes four CE runs from the experimental conditions suggested by the FCC experimental design (a–d) and three electropherograms from the experimental conditions suggested from the desirability profiler (e–g). From the runs derived from FCC conditions, c and d have acceptable (baseline) separations while the analysis times are extremely high. In experiments a and b the analysis time has been reduced considerably while the resolution R_S, especially in b, is not acceptable. In the last three electropherograms (e–g) both the analysis time and resolution have been optimized. It is obvious that the desirability profiler suggests a series of optimized experiments supporting the conclusion that it is a robust optimization technique, which is not based on chance effects. Fig. 6 also shows the desirability values for each of the seven electropherograms.

The desirability profiler resulted in good robust values. In order to suggest further experimental conditions for good separations overlay of the contours can be applied. Fig. 7 shows two overlapping contours, which result after specifying the acceptable low and high values for the responses R_S and t_2. The white space in this plot represents alternative conditions for separations with desirable specifications. In Fig. 7 the factors pH and CD concentration are kept constant at their optimum values as derived from Fig. 4b while the factors buffer and voltage are changed in their prespecified experimental region. The surface plots in Fig. 7 correspond to changes in both responses by changing buffer and voltage.

4. Conclusion

In the present study experimental design has been applied for the first time to the optimization of a peptide enantiomer separation by CE. Several re-

sponse variables were simultaneously optimized by combining a central composite design and Derringer's desirability functions. The same general procedure could be used to find the levels of the predictor variables that optimize overall response desirability in other types of designs following the same procedure: first, fit the observed characteristics of the product using an appropriate prediction equation based on the levels of the factors and second, find the levels of the factors which simultaneously produce the most desirable predicted characteristics.

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