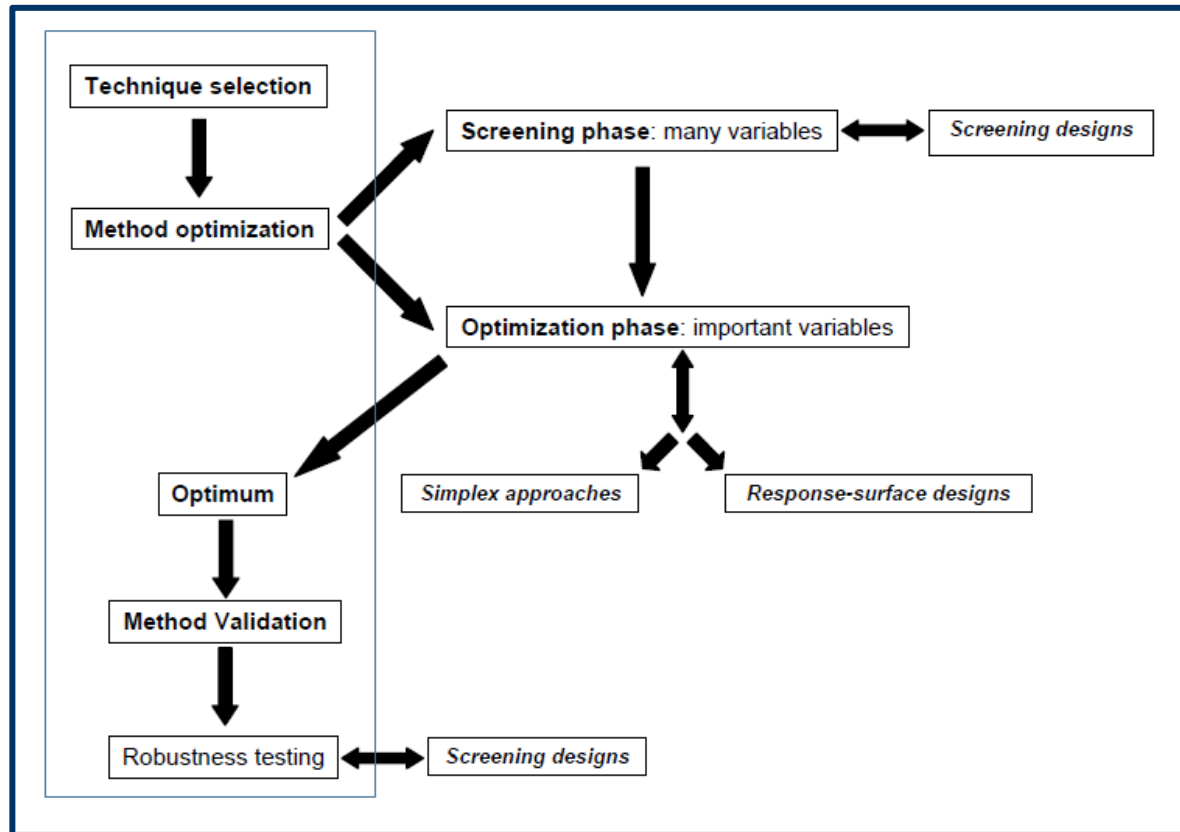


The use of Design of Experiment in Separation Science

When developing a method to separate and quantify compound(s) in a given matrix, the following **steps** are usually undertaken:

- (1) a technique is selected \Rightarrow GC, HPLC, CE, SFC, FFF;
- (2) the method is optimized in terms of resolution, sensitivity, reproducibility
- (3) the method is validated for use in routine analysis (robustness or ruggedness)

Different **DoE approaches** can be employed, according to the stage of the procedure:

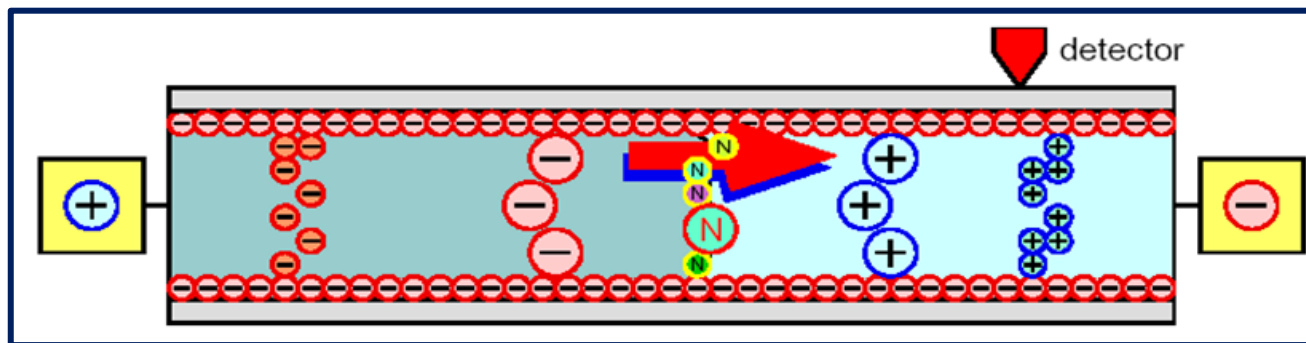
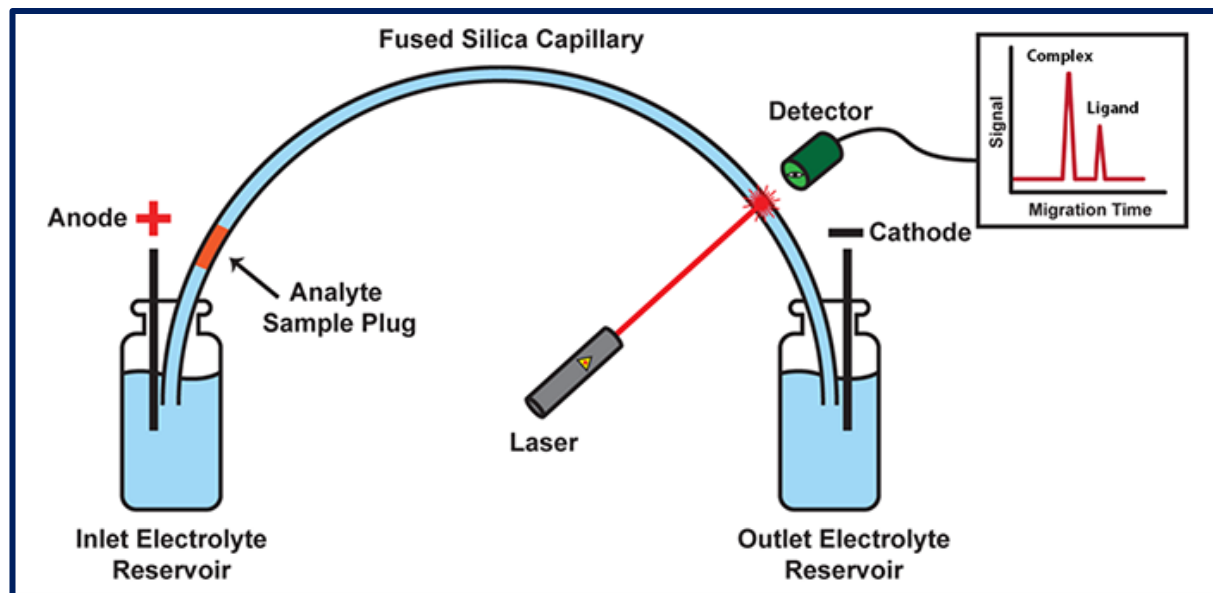


Use of screening designs

Potential **operational and environmental factors** that can be involved in the **screening designs** are summarized for **HPLC, Capillary Electrophoresys (CE) and GC** in the following table:

| <i>Operational and environmental factors</i> | | |
|---|--|---|
| <i>HPLC methods</i> | <i>CE methods</i> | <i>GC methods</i> |
| Chromatographic column: age batch length manufacturer temperature type of stationary phase Gradient elution: initial composition of mobile phase final composition of mobile phase steepness of the gradient Mobile phase: composition or % organic modifier/buffer (isocratic) concentration of additives ionic strength of the buffer buffer pH Flow rate | Additive concentrations: chiral selectors inorganic salts organic solvents surfactants Background electrolyte: electrolyte composition electrolyte concentration ionic strength of the buffer buffer pH Capillary: age batch type of coating internal diameter (i.d.) length manufacturer temperature Concentrations of rinsing liquids Rinse times Sample injection time Applied potential | Carrier gas type/composition Column: flow head pressure temperature type Detector: temperature type Injection: inlet type liner type mode (split or splitless) split flow temperature volume Temperature program: initial oven temperature final oven temperature |

A general sketch of a CE apparatus with detection based on UV absorption



Ionic species introduced into the fused silica capillary are subjected to a strong electrical field (a potential difference of several kVs can be applied between anode and cathode), causing the so-called **electrophoretic flow**, but also, in most cases, to the so-called **electroosmotic flow (EOF)**, due to the positive charges located near negatively charged silanol groups at the walls of the capillary. Neutrals (N) can move under the effect of the EOF. If the latter overcomes the electrophoretic flow, even anions are forced to move towards the cathode.

Further factors, related to signal measurement using detectors related to absorption or fluorescence spectroscopies, are usually found in the data-treatment software:

| <i>Peak-measurement/peak-analysis factors</i> |
|--|
| Detection: detection wavelength (visible, UV or fluorimetric detection) reference wavelength detection wavelength bandwidth reference wavelength bandwidth |
| Integration: sensitivity |
| Peak detection: peak width threshold |
| Signal processing: data acquisition rate type of filtering amount of filtering |

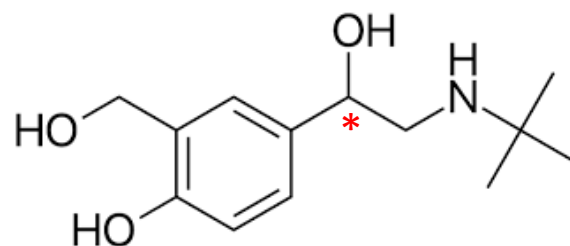
The default software settings are usually adopted by the analyst for these factors, and, apart from the detection wavelength, they are not considered for optimization.

Factors involved in screening designs are usually examined at two levels (coded values: -1 and +1), chosen on the basis of knowledge developed previously and/or of literature information. They often mark the broadest interval in which factors can be varied.

As an example, levels investigated for five factors subjected to a screening design during the development of a CE chiral separation for salbutamol, a drug used to treat asthma, are shown in the following table:

| Factor | Levels | |
|---------------------------|--------|------|
| | -1 | +1 |
| (A) MeOH (%) | 0 | 10 |
| (B) Chiral selector (%) | 0.5 | 3.0 |
| (C) Potential (kV) | 10 | 30 |
| (D) pH | 3.0 | 6.5 |
| (E) BGE concentration (M) | 0.01 | 0.05 |

BGE = background electrolyte



During **robustness testing** the examined ranges should represent the variability that can occur when transferring the method from the research to the control laboratory. As shown in the following table, **the ranges adopted for experimental parameters are usually much smaller than those evaluated during optimization**:

| Factor | Levels | | |
|---------------------------|--------|-------|-------|
| | -1 | 0 | +1 |
| (A) MeOH (%) | 4.8 | 5.0 | 5.2 |
| (B) Chiral selector (%) | 1.50 | 1.75 | 2.00 |
| (C) Potential (kV) | 22 | 24 | 26 |
| (D) pH | 5.1 | 5.3 | 5.5 |
| (E) BGE concentration (M) | 0.028 | 0.030 | 0.032 |

0 = nominal level

Note that **the nominal level (coded value = 0)** is that found after the optimization step.

Extreme levels are related to **variations of factors purposely performed to evaluate the extent of variation of the response**.

They are usually obtained as **“nominal level $\pm k \times$ uncertainty”**, where the uncertainty is the precision with which a factor can be set, whereas constant **k** is usually comprised between 2 and 10.

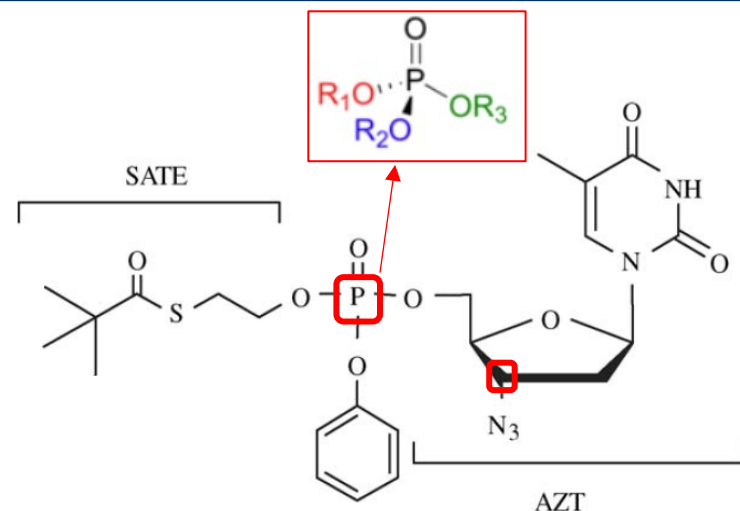
Once factors and levels are chosen, a screening based on **fractional factorial or Plackett-Burman (PB) designs** is usually performed.

As an example, the following factors/level were investigated during the screening phase in the **development of a CE method to separate pronucleotide diastereoisomers of the AZT drug in biological samples.**

| | Levels | |
|--|--------|-------|
| | -1 | +1 |
| (A) Chiral additive concentration [CM- β -CD] (mM) | 5 | 15 |
| (B) Buffer concentration (mM) | 50 | 100 |
| (C) Percent MeOH (v/v) | 0 | 10 |
| (D) Volume injected (nL) | 4.64 | 12.38 |
| (E) Capillary length (cm) | 31.2 | 51.2 |
| (F) Potential (V cm^{-1}) | 0.50 | 0.80 |
| (G) Capillary temperature ($^{\circ}\text{C}$) | 15 | 25 |

(-1) and (+1) = extreme levels

CM- β -CD = carboxymethyl- β -cyclodextrin



Note that pronucleotides are lipophilic prodrugs that can penetrate the cell membrane and deliver an active monophosphorylated nucleoside to be exploited as an antiviral drug.

In the specific example, the reported **derivative of 3'-azido-2',3'-dideoxythymidine (AZT) including a S-pivaloyl-2-thioethyl (an example of S-acyl-2-thioethyl, SATE, protecting groups) and an aryl residue as biolabile phosphate protectors, represents a couple of diastereoisomers, since C 3' and P atoms are chiral centers.**

A twelve-experiment Plackett-Burman (PB) design was adopted. Since the number of factors potentially examined in such a PB design ($N-1 = 11$) exceeded that of really tested factors, four dummy factors (d_1, \dots, d_4), i.e., hypothetic variables for which the change between coded levels -1 and +1 has no physicochemical meaning, were introduced.

Three responses, referred to the couple of diastereoisomers, were considered:

- 1) selectivity, S
- 2) resolution, $R_s = 2 \left(\frac{t_2 - t_1}{\omega_1 + \omega_2} \right)$
- 3) analysis time, t

The results reported in the table were obtained:

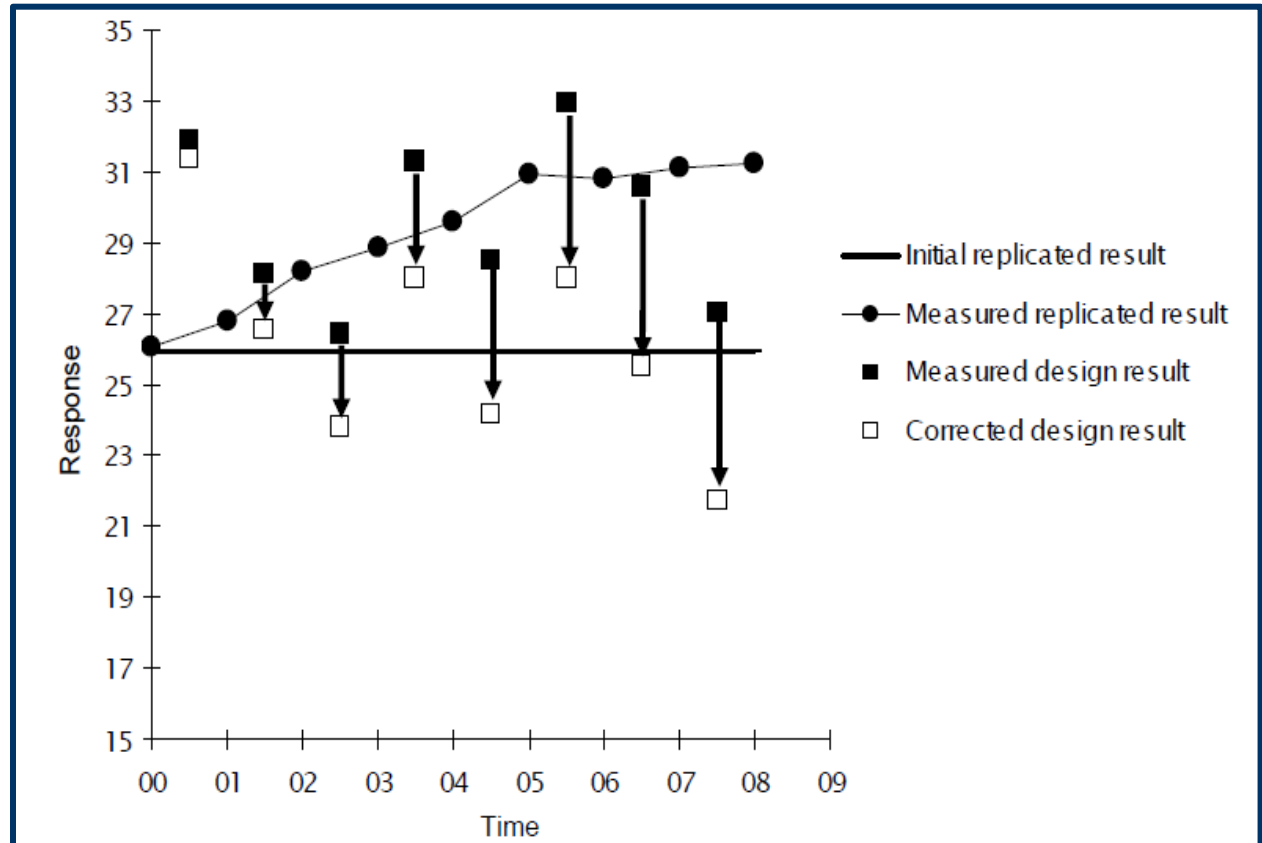
| Exp. | Factors | | | | | | | | | | | Responses | | |
|------|---------|----|----|----|----|----|----|----------------|----------------|----------------|----------------|-----------|----------------|---------|
| | A | B | C | D | E | F | G | d ₁ | d ₂ | d ₃ | d ₄ | S | R _s | t (min) |
| 1 | -1 | 1 | -1 | -1 | -1 | 1 | 1 | 1 | -1 | 1 | 1 | 1.12 | 1.91 | 4.02 |
| 2 | 1 | -1 | 1 | -1 | -1 | -1 | 1 | 1 | 1 | -1 | 1 | 1.05 | 1.69 | 10.15 |
| 3 | 1 | 1 | -1 | 1 | -1 | -1 | -1 | 1 | 1 | 1 | -1 | 1.05 | 1.17 | 14.50 |
| 4 | -1 | 1 | 1 | -1 | 1 | -1 | -1 | -1 | 1 | 1 | 1 | 1.12 | 4.30 | 26.70 |
| 5 | 1 | -1 | 1 | 1 | -1 | 1 | -1 | -1 | -1 | 1 | 1 | 1.06 | 1.45 | 6.53 |
| 6 | 1 | 1 | -1 | 1 | 1 | -1 | 1 | -1 | -1 | -1 | 1 | 1.05 | 1.76 | 22.85 |
| 7 | 1 | 1 | 1 | -1 | 1 | 1 | -1 | 1 | -1 | -1 | -1 | 1.06 | 2.76 | 19.41 |
| 8 | -1 | 1 | 1 | 1 | -1 | 1 | 1 | -1 | 1 | -1 | -1 | 1.13 | 1.10 | 4.49 |
| 9 | -1 | -1 | 1 | 1 | 1 | -1 | 1 | 1 | -1 | 1 | -1 | 1.11 | 1.81 | 12.31 |
| 10 | -1 | -1 | -1 | 1 | 1 | 1 | -1 | 1 | 1 | -1 | 1 | 1.10 | 2.33 | 7.74 |
| 11 | 1 | -1 | -1 | -1 | 1 | 1 | 1 | -1 | 1 | 1 | -1 | 1.05 | 1.79 | 7.52 |
| 12 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 1.10 | 2.05 | 8.71 |

Note: it is often recommended that experiments are performed in a random sequence, to minimize uncontrolled effects on the estimated effects.

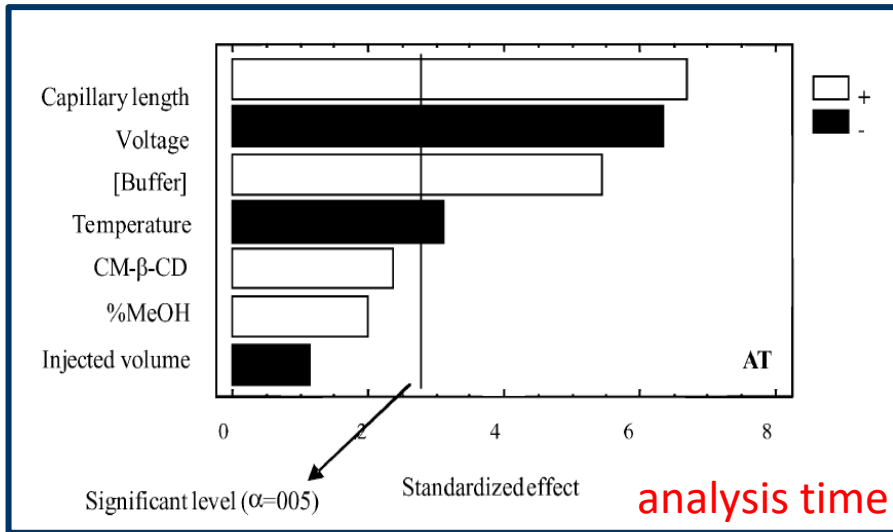
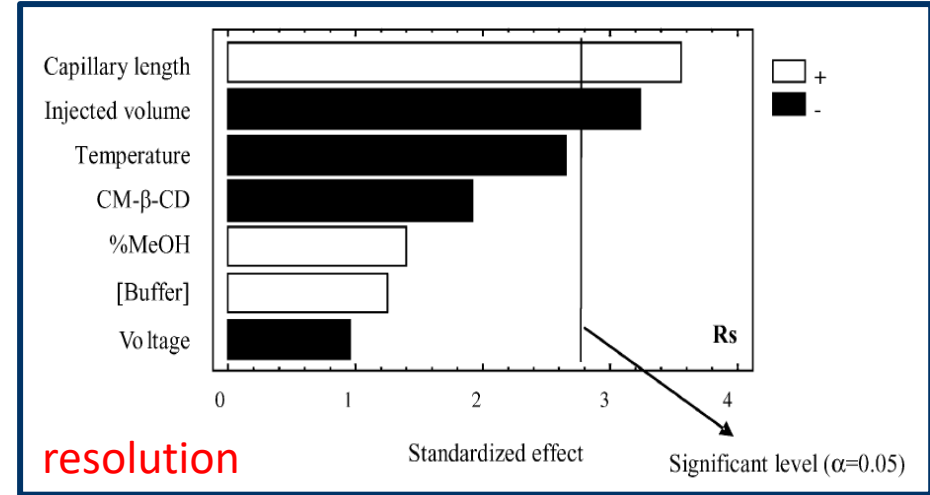
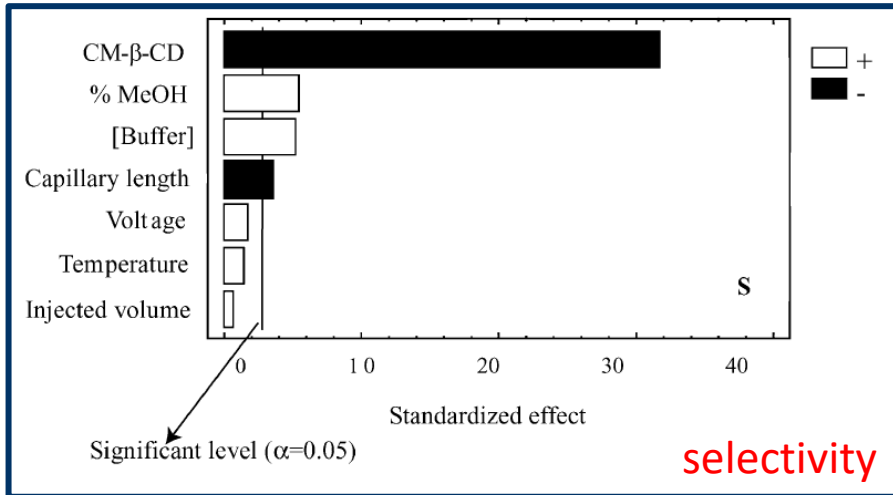
If the presence of time or drift effects, i.e., of response variations with time, is suspected, special control measurements can be done to account for them, as described by the following figure.

Black circles in the figure represent replicated responses, i.e., responses obtained at different times, with factors having the same levels.

It is apparent that a drift occurs in the response; it can be exploited to correct results referred to the experimental design, according to the time when each of the latter is obtained.



Pareto charts were obtained to evaluate the significance of effects due to each of the seven investigated factors on the three responses:



As expected, the concentration of β -cyclodextrin was found to influence selectivity in a significant extent.

Resolution was increased by the increase of capillary length and by the decrease of injection volume.

Analysis time was lowered significantly by the increase of the capillary voltage, but it was increased remarkably by the increase of capillary length, as expected.

Based the results of the screening study, analytical parameters were initially set as follows:

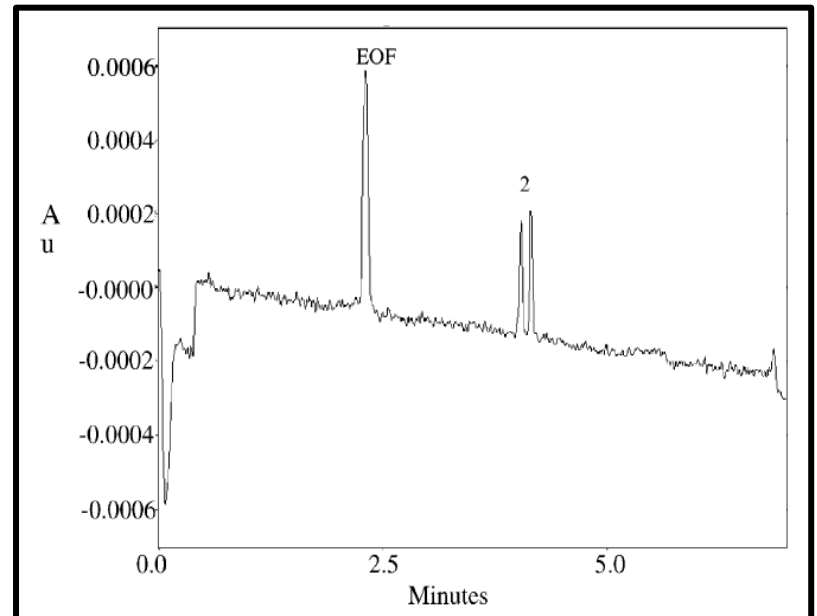
- A) 5 mM CM- β -CD
- B) 50 mM phosphate buffer (adjusted to pH 6.2 with triethanolamine)
- C) 0% methanol
- D) 4.64 nL for volume injected
- E) 31.2 cm for capillary length
- F) 0.8 V/cm for the potential (actually, for the electric field)
- G) 15 °C temperature

Under these conditions (with detection performed by absorption at 268 nm) the separation was quite rapid (less than 3.5 min) but unable to fully resolve the target couple of diastereoisomers.

This effect was related to the fact that a screening design assumes a linear dependence of response from the values of a factor, yet this assumption can be not true.

In the specific case, the dependence of selectivity of diastereoisomers/enantiomers on the concentration of chirality inducers often passes through a maximum within the explored range.

Specific measurements indicated that 8 mM CM- β -CD was the best concentration in terms of separation between diastereoisomers.

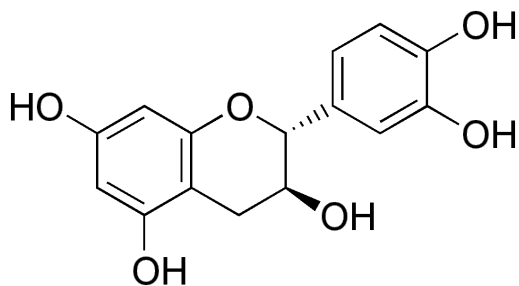


Optimization based on the simplex approach

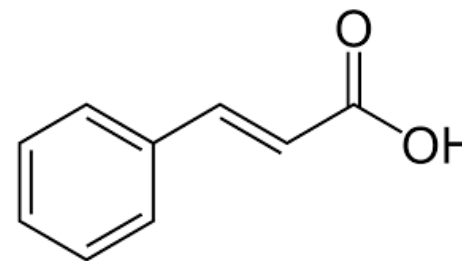
Either sequential optimization methods, like simplex approaches, or simultaneous optimization strategies, using response-surface designs, are usually applied during the development of separation methods.

As an example of simplex optimization, the HPLC separation based on C18 columns for nine phenolic compounds occurring in vegetation waters and sugar beet vinasse was reported:

- a) 3,4,5 – trihydroxy-benzoic (gallic) acid
- b) (+)-catechin
- c) 4-hydroxy-benzoic acid
- d) 3,4- dihydroxybenzoic acid
- e) 4-hydroxy-3,5-methoxybenzoic (syringic) acid
- f) 4-hydroxy-cinnamic (*p*-coumaric) acid
- g) 3-methoxy-4 hydroxycinnamic (ferulic) acid
- h) 3,5-methoxy-4-hydroxy-cinnamic (synaptic) acid
- i) 4-methoxy-benzoic (anisic) acid

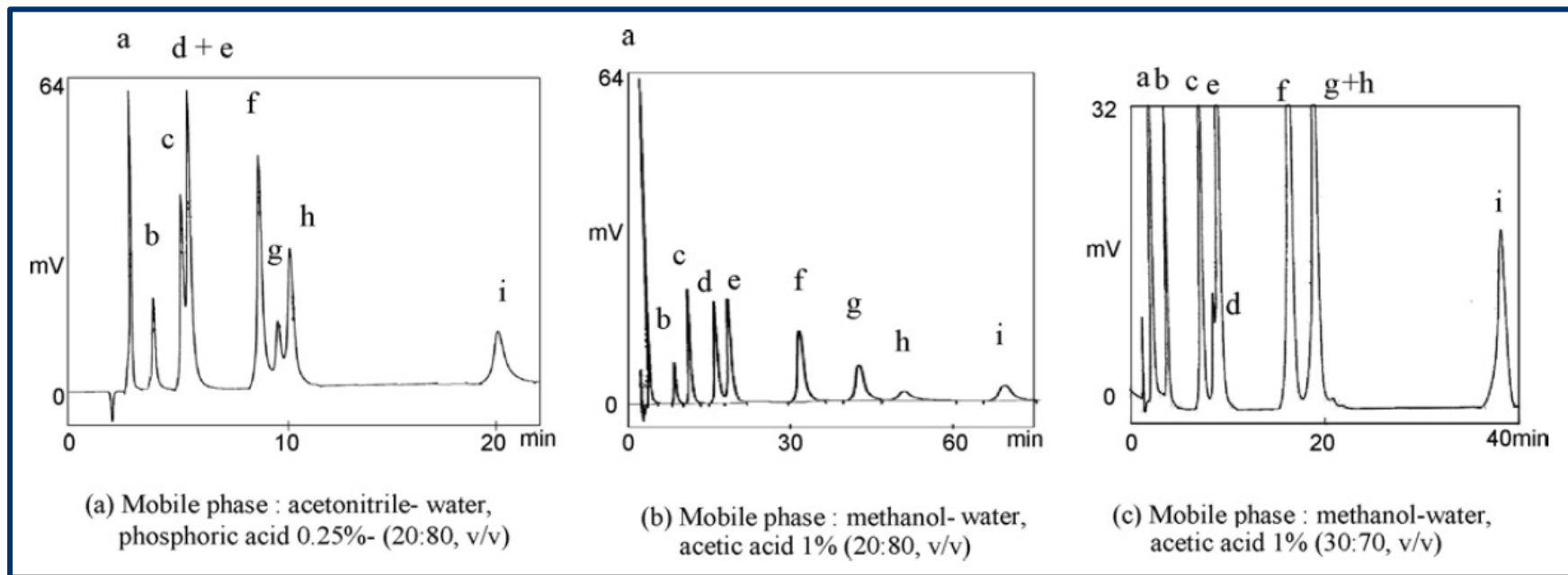


(+)-catechin



cinnamic acid

The study's authors first checked the eluent strength of different mobile phases under isocratic conditions:



Methanol was chosen as organic modifier, since it generally provided a better resolution, although with a longer separation time, already under isocratic conditions.

In order to optimize gradient elution, the following four factors were considered:

- A) Initial methanol percentage;
- B) Duration of the initial isocratic segment;
- C) Gradient slope (up to 30% methanol);
- D) column temperature

Reference values, i.e., values of factors corresponding to the initial reduced co-ordinates (all 0 values) of the domain center in the simplex hyperspace, were defined for the four factors as follows:

- A) 15% methanol (a lower percentage would have led to too long analysis times)
- B) 18 min (5 compounds were well resolved in the run described in panel b of the previous figure in a time range of 18.7 min)
- C) 0.68 % min⁻¹
- D) 25°C

The level for a *j*-th variable in a *i*-th subsequent experiment during simplex operation was calculated with the following equation:

$$x_{i,j} = x_{0,j} + X_{i,j} \times \Delta_{xj}$$

where:

$x_{0,j}$ represents the *j*-th variable level in the first experiment

$X_{i,j}$ is the reduced co-ordinate for that variable in the *i*-th simplex experiment

Δ_{xj} is the simplex step size for the *j*-th variable

In the present case a variable-size simplex was adopted.

In order to find a single parameter describing the quality of separation, representing the response to be optimized, a **Chromatographic Response Function (CRF)** was adopted:

$$\text{CRF} = \sum_{i=1}^{p-1} R_i + aP + b(t_M - t_L)$$

where:

R_i is the resolution between adjacent peak pairs

P is the number of peaks detected

t_M is the target retention time in minutes

t_L is the retention time for the last peak,

a and b are two arbitrary weighting factors, set equal to 1 and 0.5, respectively.

During the study the minimum required resolution between all peak pairs was set as 2, whereas the target retention time was set as 45 min.

The optimum value of CRF was thus equal to 25.

Moreover, if R_i values were higher than 2, this value was attributed to them, so that too high values of resolution for specific couples of peaks could not compensate for low values referred to other couples of peaks.

A summary of levels assumed by the four variables and responses obtained during the subsequent steps of the simplex evolution is reported in the following table:

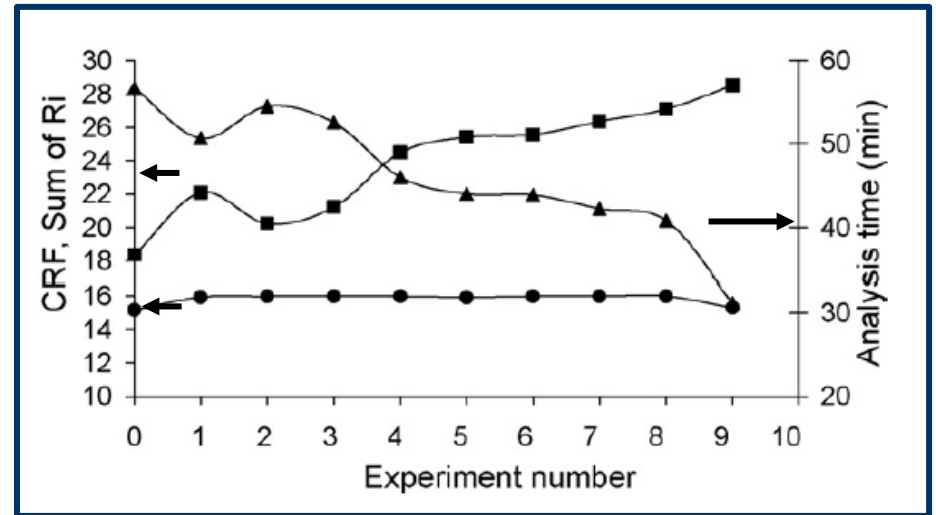
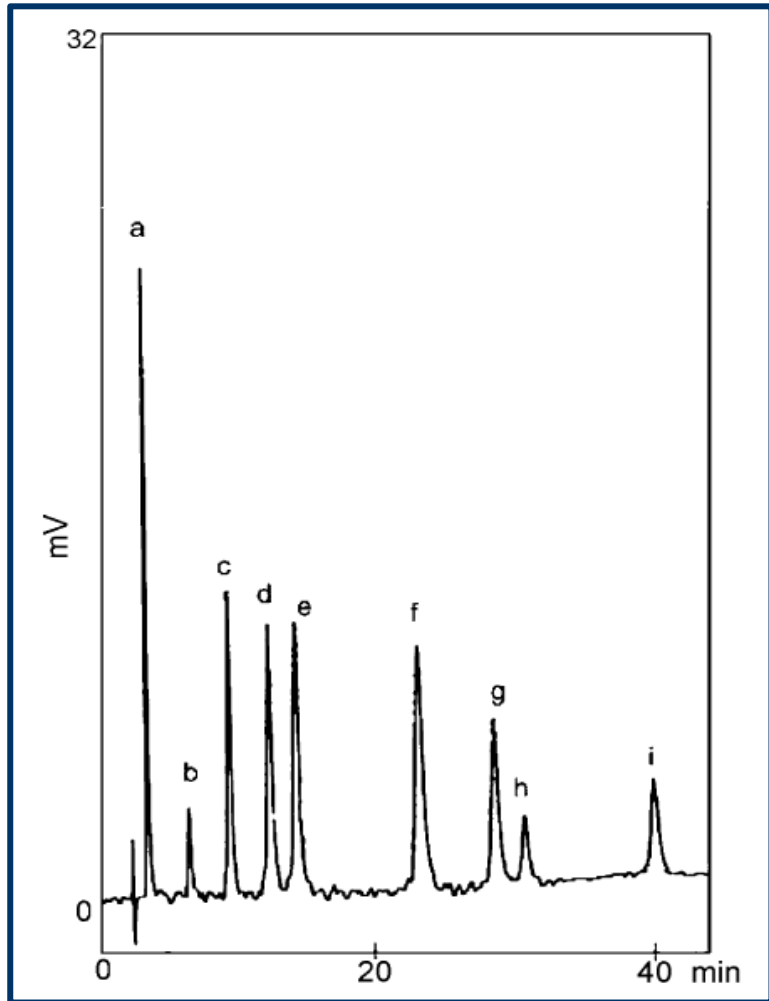
| Experiment number | MeOH (%) | t_i (min) | Gradient (% min ⁻¹) ^a | Temperature (°C) | Time of gradient (min) | $\sum_i R_i$ | t_L (min) | CRF | Number simplex | | | | | |
|-------------------|----------|-------------|--|------------------|------------------------|--------------|-------------|-------|----------------|-------|-------|-------|-------|-------|
| | | | | | | | | | 1 | 2 | 3 | 4 | 5 | 6 |
| 0 | 15 | 18.00 | 0.68 | 25 | 22.1 | 15.2 | 56.70 | 18.34 | 5 (w) | | | | | |
| 1 | 18 | 18.00 | 0.68 | 25 | 17.6 | 16.0 | 50.70 | 22.08 | 2 | 3 | 4 | 5 (w) | | |
| 2 | 17 | 21.12 | 0.68 | 25 | 19.9 | 16.0 | 54.50 | 20.23 | 4 | 5 (w) | | | | |
| 3 | 17 | 19.04 | 0.79 | 25 | 17.1 | 16.0 | 52.57 | 21.21 | 3 | 4 | 5 (w) | | | |
| 4 | 17 | 19.04 | 0.71 | 29 | 19.1 | 16.0 | 46.02 | 24.49 | 1 (b) | 2 | 3 | 4 | 5 (w) | |
| 5 | 19 | 20.60 | 0.75 | 27 | 15.0 | 16.0 | 44.02 | 25.42 | | 1 (b) | 2 | 3 | 4 | 5 (w) |
| 6 | 18 | 17.22 | 0.78 | 28 | 14.8 | 16.0 | 43.87 | 25.56 | | | 1 (b) | 2 | 3 | 4 |
| 7 | 19 | 18.39 | 0.67 | 29 | 16.0 | 16.0 | 42.27 | 26.36 | | | | 1 (b) | 2 | 3 |
| 8 | 18 | 19.63 | 0.78 | 32 | 14.9 | 16.0 | 40.80 | 27.10 | | | | | 1 (b) | 2 |
| 9 | 21 | 19.00 | 0.78 | 29 | 11.6 | 15.3 | 31.16 | 28.51 | | | | | | 1 (b) |

^a MeOH ending 30%.

The classification of the five points of each subsequent simplex in terms of response is reported in the last section of the table, with symbols w and b referred to the worst and the best point, respectively.

As apparent, one of the first experiments, #4, already provided a CRF value close to the optimum, with resolution equal to 2 for all peak pairs. However, a further improvement, in terms of total analysis time, was searched for and the procedure was stopped at experiment #9, with the last retention time being ca. 31 min.

Actually, the sum of resolutions was not optimal (i.e., lower than 16) for experiment #9, thus experiment #8 was chosen as the optimal one:



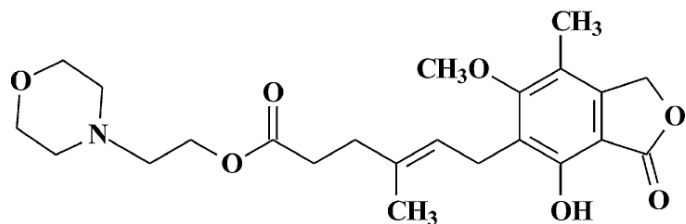
The chromatogram shown in the figure was the one resulting from conditions of **experiment #8**.

Optimization based on response surface designs

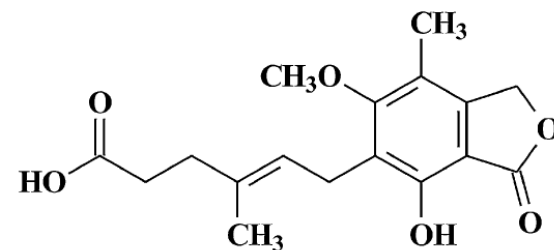
Two or three factors found to be most important after the screening step are usually taken into account for response surface designs. They are examined at three or more levels, to enable modelling of curvature in the response surface.

As for responses, the separation of a critical peak pair and the run time of an analysis are often selected.

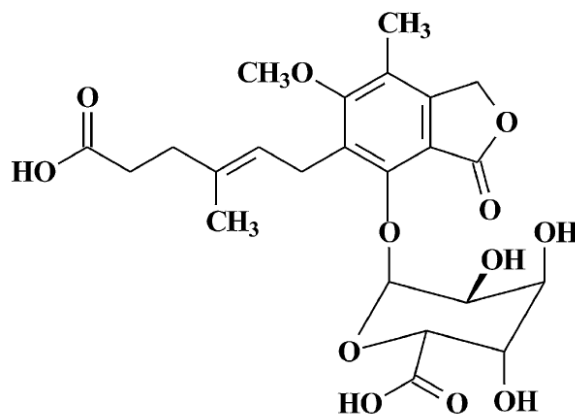
As an example, the optimization of an isocratic reversed-phase HPLC method for the simultaneous determination of mycophenolic acid (MPA), i.e., the active form of the immunosuppressive agent known as mycophenolate mofetil, and of its glucuronide (MPAG) in human urine and plasma will be presented.



mycophenolate mofetil



mycophenolic acid



mycophenolic acid glucuronide

A fractional factorial design was preliminarily adopted to evaluate the effect of the following factors: pH of the aqueous phase (a 40 mM KH_2PO_4 solution), percentage of acetonitrile, column temperature and flow rate on the retention time and retention factor for MPA and MPAG, respectively.

The pH of aqueous phase and the acetonitrile percentage were found to have a significant effect.

A face centered Central Composite Design (FC-CCD) was built from a full 2^k factorial design, to which a star design and a center point were added. Four replicates were performed on the center point, thus 12 experiments (4 + 4 + 4) were required.

A table summarizing coded values of the two variables and obtained responses is shown on the right:

Values -1, 0 and 1 for the two variables correspond to:

pH) 2.4, 3.6, 4.8

acetonitrile percentages) 25, 30, 35%

| Experiment no. | Variables | | Investigated responses | |
|----------------|-----------|----|------------------------|--------------------------|
| | A | B | MPA Retention time | MPAG Retention factor |
| 1 | +1 | +1 | 1.084 | 0.08 |
| 2 | -1 | +1 | 1.266 | 0.299 |
| 3 | +1 | -1 | 3.854 | 0.33 |
| 4 | -1 | -1 | 4.972 | 1.11 |
| 5 | +1 | 0 | 1.778 | 0.11 |
| 6 | -1 | 0 | 2.37 | 0.6 |
| 7 | 0 | +1 | 1.359 | 0.233 |
| 8 | 0 | -1 | 5.123 | 0.89 |
| 9 | 0 | 0 | 2.8 | 0.5 |
| 10 | 0 | 0 | 2.4 | 0.54 |
| 11 | 0 | 0 | 3 | 0.525 |
| 12 | 0 | 0 | 3 | 0.53 |

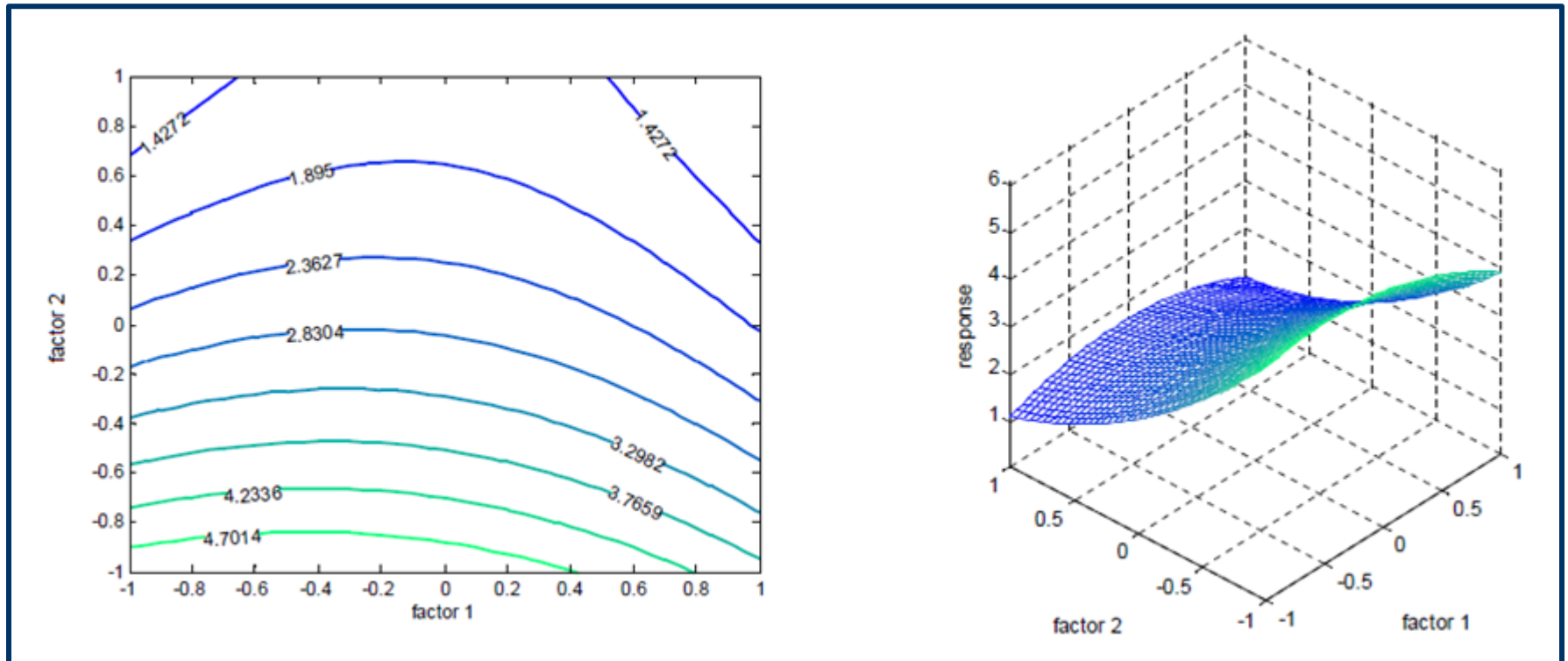
A – pH value of the phosphate buffer (KH_2PO_4); B – acetonitrile in the mobile phase (%).

After quadratic regression on the model coefficients, the following polynomial equations were obtained for the two compounds:

MPA)
$$y = 2.75 - 0.32x_1 - 1.07x_2 + 0.23x_1x_2 - 0.59x_1^2 + 0.58x_2^2$$

MPAG)
$$y = 0.51 - 0.25x_1 - 0.29x_2 + 0.14x_1x_2 - 0.14x_1^2 + 0.07x_2^2$$

As an example, the 2D contour plot and the 3D response-surface referred to the MPA retention time are reported in the figure below:



In order to reach a compromise among responses which could better satisfy the goals, the **Derringer's desirability function**, D , was used, thus converting a multi-response problem into a single response one.

By definition, D is defined as the geometric mean, weighted or not, of the individual desirability functions, d_i :

$$D = [d_1^{p_1} \times d_2^{p_2} \times d_3^{p_3} \times \dots \times d_n^{p_n}]^{1/n}$$

where p_i represent the weights of different responses.

When the goal is to **maximize a response**, each d_i is defined as follows:

$$d_i = \left[\frac{Y_i - \text{Low}_i}{\text{High}_i - \text{Low}_i} \right]$$

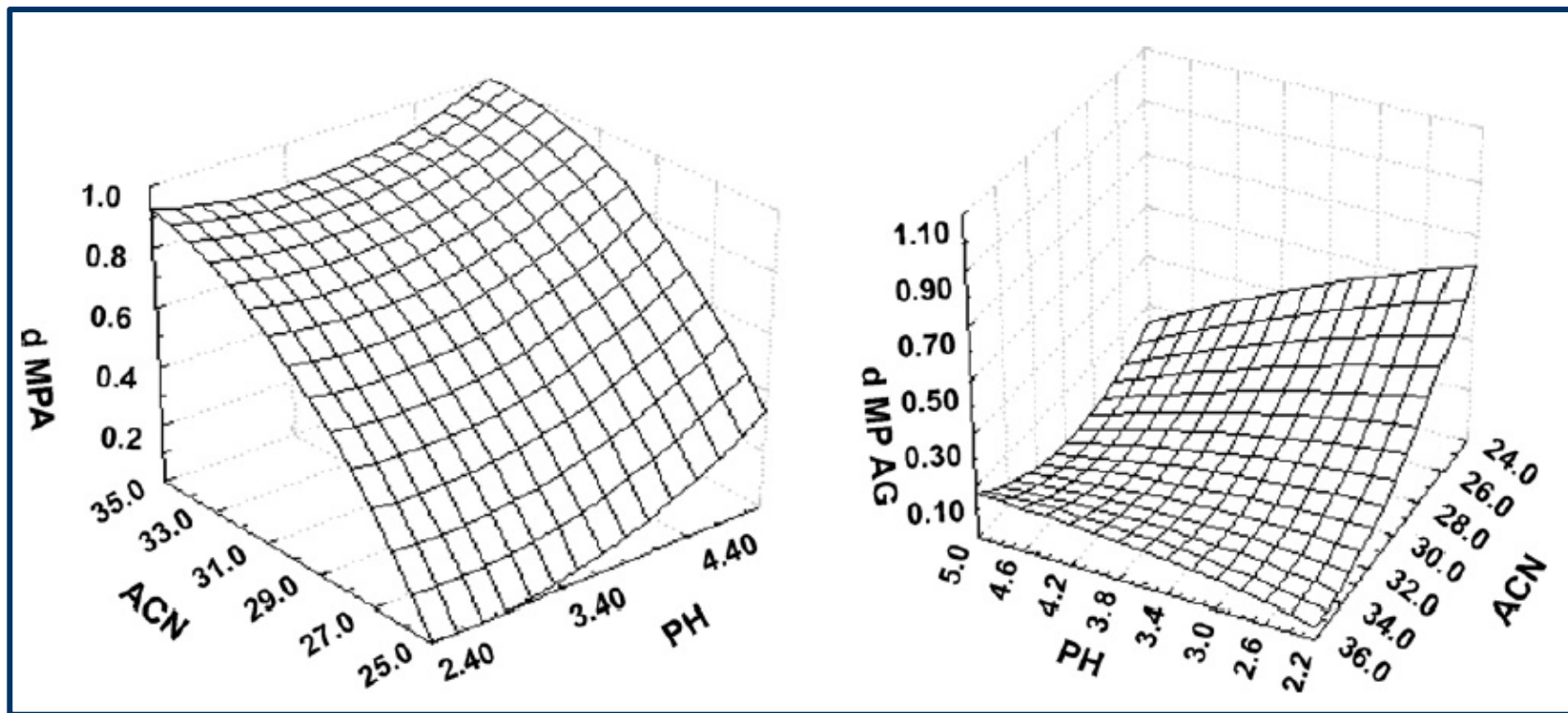
where Y_i is the predicted response using the fitted model, High_i and Low_i represent the highest and the lowest values obtained for the i -th response, respectively.

If the goal is to **minimize a response**, d_i is defined as: $d_i = \left[\frac{\text{High}_i - Y_i}{\text{High}_i - \text{Low}_i} \right]$

In any case, d_i is equal to 0 for a completely undesired response and to 1 for a fully desired response.

In the present case the lower and upper limits set for the MPA retention time were 0.96 and 5.04 min, respectively, whereas they were 0.8 and 1.12 for the retention factor of MPAG.

Response surface plots obtained for the single desirability functions were:



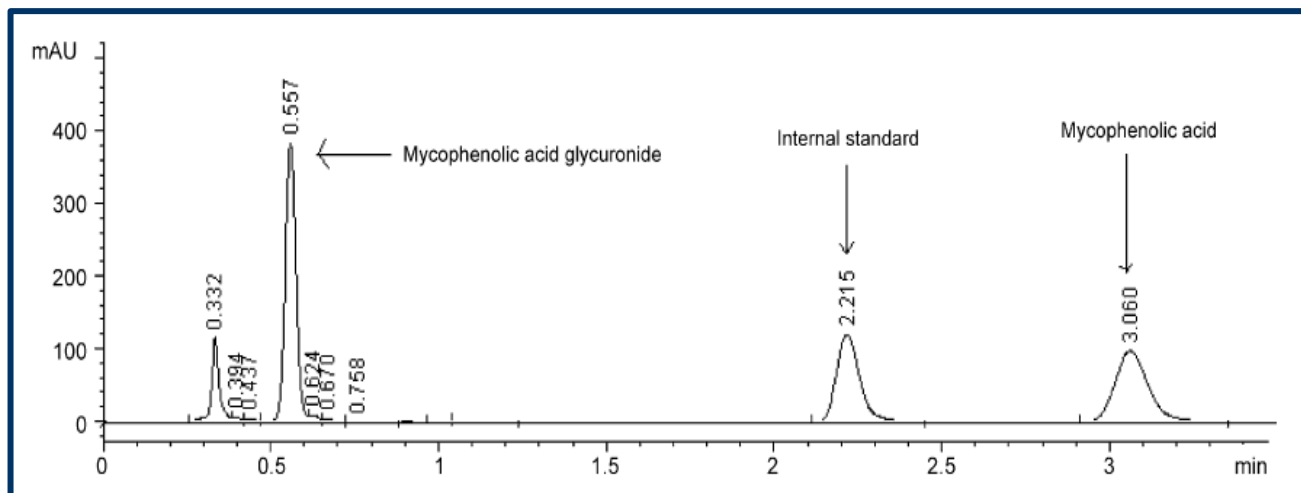
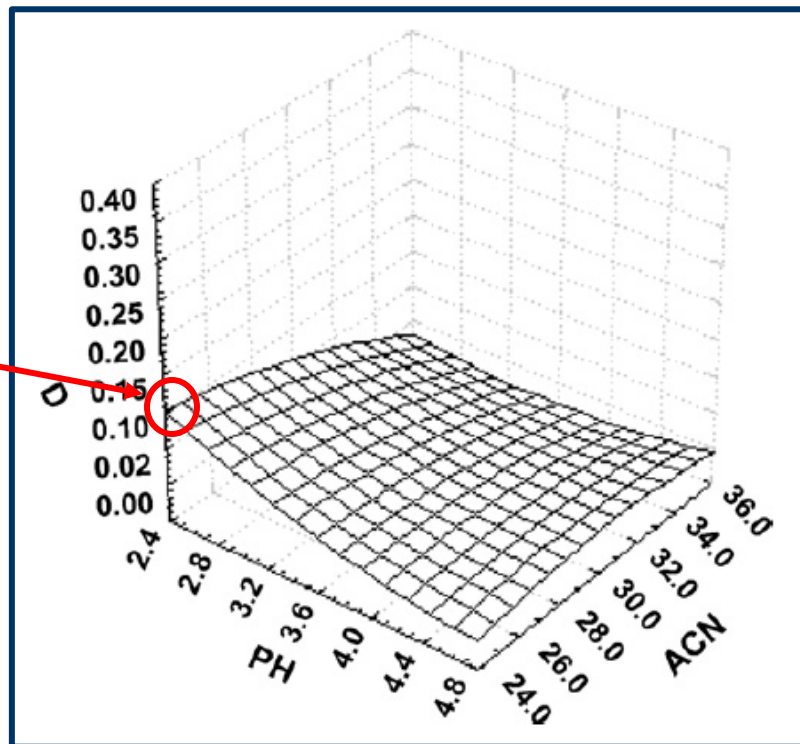
As apparent, d_{MPA} approaches to 1 when the percentage of acetonitrile approaches to 35% and the pH to 4.40. The interaction between the two factors is present, since the influence of aqueous phase pH is more evident at low acetonitrile percentages.

An interaction between factors can be inferred also in the case of d_{MPAG} , with the influence of acetonitrile percentages much more evident at low pH values.

The graphical representation of the Derringer's desirability function, considering a unitary weight for d_{MPA} and d_{MPAG} , is shown in the figure on the right:

A pH 2.4 for the aqueous phase and a 24% percentage for acetonitrile were finally adopted as the optimal values of the two parameters.

A representative chromatogram obtained for a plasma sample spiked with 10 $\mu\text{g/mL}$ of MPA, 20 $\mu\text{g/mL}$ of MPAG and 50 $\mu\text{g/mL}$ of internal standard (the propyl ester of p-hydroxy-benzoic acid) is shown below:

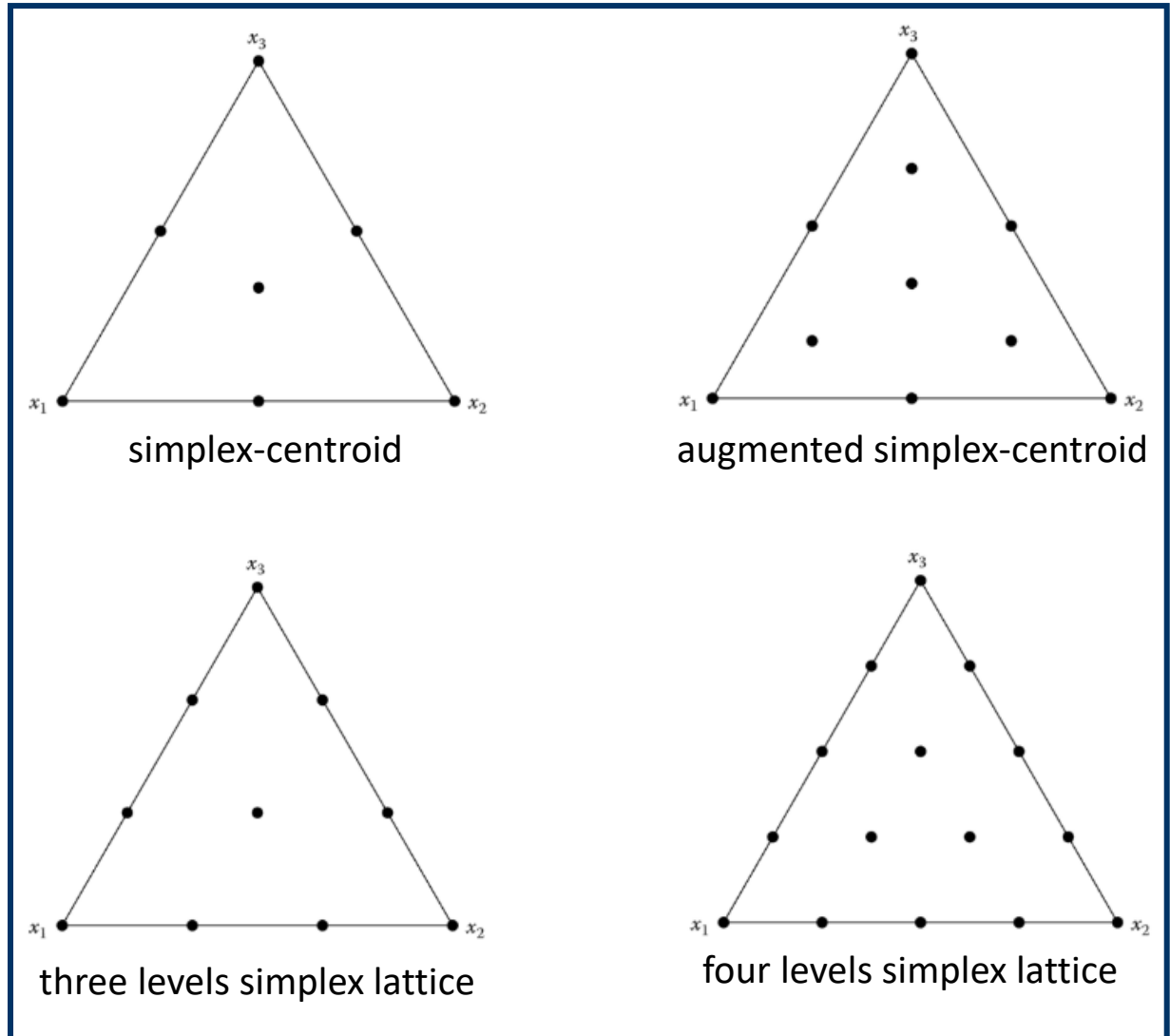


Application of Mixture Design to optimization of HPLC mobile phases

Mixture design can be conveniently applied to the optimization of the composition of mobile phases in liquid chromatography.

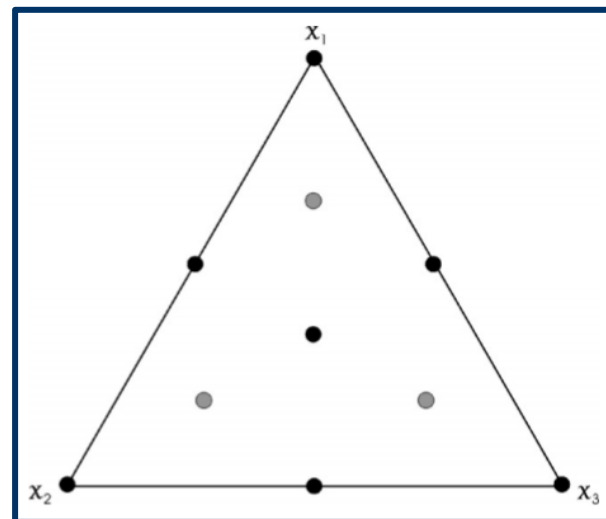
When three constituents are considered for a mobile phase, mixture designs can be represented using a triangle, as shown in the figure:

Each point at a vertex of the triangle represents a pure component or a mixture of components. The points centered on each side of the triangle represent 1:1 binary mixtures of the components or mixtures of their neighboring vertex points. The point in the center of the triangle represents a 1:1:1 ternary mixture of the three pure components or mixtures.



In the **simplex centroid design with axial points**, that is also used for mobile phase optimization, **axial points**, corresponding to combinations with **2/3** portion of one of the ingredients and **1/6** portions of the other two, are also considered.

They are represented as **grey points** in the figure:



As an example, the table on the right reports the proportions of acetonitrile (ACN), methanol (MeOH) and tetrahydrofuran (THF) adopted for a simplex centroid design with axial points.

Resolutions reported in the last columns are **replicated values** obtained for the separation of two peaks in the HPLC separation.

| Mixture | x_{ACN} | x_{MeOH} | x_{THF} | Resolution |
|---------|------------------|-------------------|------------------|------------|
| 1 | 1 | 0 | 0 | 0.99, 1.07 |
| 2 | 0 | 1 | 0 | 5.31, 5.64 |
| 3 | 0 | 0 | 1 | 4.12, 4.34 |
| 4 | 0.5 | 0.5 | 0 | 3.79, 3.98 |
| 5 | 0.5 | 0 | 0.5 | 3.88, 4.07 |
| 6 | 0 | 0.5 | 0.5 | 5.85, 6.16 |
| 7 | 0.333 | 0.333 | 0.333 | 5.22, 5.21 |
| 8 | 0.667 | 0.167 | 0.167 | 3.42, 3.50 |
| 9 | 0.167 | 0.667 | 0.167 | 5.80, 5.81 |
| 10 | 0.167 | 0.167 | 0.667 | 4.84, 4.83 |

In the application, a linear model was first adjusted to the experimental data referred only to the simplex centroid points, each obtained in duplicate. Axial points were temporarily removed from the data set for model determination, so they could be used for model validation.

The following model was then obtained:

$$\hat{y} = 1.667x_{\text{ACN}} + 6.035x_{\text{MeOH}} + 5.075x_{\text{THF}}$$

ANOVA was adopted to evaluate the lack-of-fit:

| Variation | SS | df | MS | F-test quotient |
|--------------|---------|----|---------|-----------------|
| Linear model | | | | |
| Regression | 26.3462 | 2 | 13.1731 | 24.16 |
| Residual | 5.9977 | 11 | 0.5452 | – |
| Lack of fit | 5.8316 | 4 | 1.4579 | 61.46 |
| Pure error | 0.1661 | 7 | 0.0237 | – |
| Total | 32.3439 | 13 | 2.4880 | |

Since the F-test quotient $MS_{\text{lack-of-fit}}/MS_{\text{pure error}}$, 61.46, was much higher than the $F_{4,7}$ critical value at 95% confidence (4.12), a clear evidence of lack of fit was obtained, thus a model including interaction terms was adopted:

$$\hat{y} = 1.013x_{\text{ACN}} + 5.458x_{\text{MeOH}} + 4.213x_{\text{THF}} + 2.865x_{\text{ACN}}x_{\text{MeOH}} + 5.715x_{\text{ACN}}x_{\text{THF}} + 4.945x_{\text{MeOH}}x_{\text{THF}}$$

The resulting ANOVA table was the following:

| Variation | SS | df | MS | F-test quotient |
|-----------------|---------|----|--------|-----------------|
| Quadratic model | | | | |
| Regression | 32.1038 | 5 | 6.4208 | 213.98 |
| Residual | 0.2401 | 8 | 0.0300 | – |
| Lack of fit | 0.0740 | 1 | 0.0740 | 3.12 |
| Pure error | 0.1661 | 7 | 0.0237 | – |
| Total | 32.3439 | 13 | | |

In this case the F-test quotient $MS_{\text{lack-of-fit}}/MS_{\text{pure error}}$, 3.12, was lower than the $F_{1,7}$ critical value at 95% confidence (5.59), thus no evidence of lack of fit was present.

The regression significance was then evaluated using the $MS_{\text{Regression}}/MS_{\text{Residual}}$ ratio, 213.98, which was much higher than the $F_{5,8}$ critical value (3.69), thus indicating a highly significant regression.

The model was finally refined including also axial points, thus obtaining the following equation:

$$\begin{aligned}\hat{y} = & 1.031x_{\text{ACN}} + 5.527x_{\text{MeOH}} + 4.130x_{\text{THF}} + 3.061x_{\text{ACN}}x_{\text{MeOH}} \\ & + 5.605x_{\text{ACN}}x_{\text{THF}} + 4.937x_{\text{MeOH}}x_{\text{THF}}\end{aligned}$$

According to the model, methanol and tetrahydrofuran were found to be more efficient in separating the two peaks, whereas acetonitrile did not seem to be particularly effective. Synergic interactions between methanol or acetonitrile and THF were found to be quite relevant.

The response contour plot arising from the model is shown in the figure on the right:

According to the model, the best resolution can be obtained using binary mixtures containing almost equal volumes of methanol and THF.

As apparent, the presence of acetonitrile in the mixture leads to a decrease in the achieved resolution.

