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# Fast generic chiral separation strategies using electrophoretic and liquid chromatographic techniques

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**Abstract**

Because of the large number of commercially available chiral selectors both for electrophoretic and chromatographic techniques, the experimental possibilities to separate enantiomers are numerous. As a result, the development of a proper separation method for a chiral molecule is challenging. Generic separation strategies can present a useful approach for chiral method development. This paper overviews some strategies that have been developed earlier for different electrophoretic and chromatographic techniques. These strategies can be used either for impurity determination of enantiomers or for the enantioseparation of racemic mixtures. They are not only generic, i.e. applicable on diverse molecules, but are also fast, i.e. requiring only a limited number of experiments to reach a decision.


*Keywords:* Chiral separations; Generic separation strategies; Method development; Capillary electrophoresis; Liquid chromatography

## 1. Introduction

In the last decades, chirality has become a major concern in pharmaceutical industry. New chiral drug substances have to be stereochemically identified before being marketed since enantiomers can differ in pharmacological, pharmacokinetic and toxicological properties when exposed to chiral environments such as biological systems, e.g. the human body [1–3]. In such systems, one enantiomer (the eutomer) will be therapeutically active while the other, the distomer, can be less active or inactive, it can have an opposite or even toxic effect. Obviously the separation of enantiomers is gaining importance at almost every level of pharmaceutical drug development and quality assurance [4].

Several analytical techniques can be used to separate enantiomers but the most frequently used in pharmaceutical analysis are capillary electrophoresis (CE) and high-performance liquid chromatography (HPLC) [5,6]. Separations can be performed either directly or indirectly. The indirect approach uses a reaction of an enantiomerically pure chiral derivatization agent with the enantiomers to form diastereomers. These can be separated with any conventional achiral separation system since diastereomers differ in physico-chemical properties. The direct approach for chiral separations consists of the formation of transient diastereomeric complexes between the enantiomers and a chiral selector. Chiral selectors can either be added to the mobile phase or the background electrolyte, or bonded onto a chromatographic support creating a chiral stationary phase (CSP).

The fact that nowadays many chiral selectors are available makes the selection of proper analytical conditions to obtain an acceptable result for a given analyte, challenging. Often the search for suitable conditions is still a trial-and-error approach which can be extensively time-, money- and labor consuming. To avoid these drawbacks, fast generic chiral separation strategies have been created [7–13]. A fast generic strategy allows developing separations for large sets of substances with different molecular structure in a limited number of experiments.

In this paper we present an overview of such strategies in CE and in HPLC. For the latter technique, strategies in normal-phase liquid chromatography (NPLC), reversed-phase liquid chromatography (RPLC) and polar organic solvent chromatography (POSC) have been defined. These strategies can be considered complementary and are used as alternatives when one fails to separate a given compound.

The first step in the strategies is a screening where the analytes are examined according to a limited number of conditions.

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After evaluating the resolution (Rs), which is the most important response for chiral separations, some optimization steps can be considered when desired. When compounds have a Rs > 1.5 after screening, i.e. are baseline separated, usually no further optimization is needed but the strategies allow optimizing responses such as analysis time or peak shape. When 0 < Rs < 1.5 after screening, i.e. when the peaks are only partly separated, some optimization should be performed to increase the resolution. However when Rs = 0 after screening, a limited number of additional experiments will be done but when the separation does not improve, the applied technique or mode is changed.

When peaks become baseline separated (Rs > 1.5) after execution of the additional experiments, again there is the possibility to optimize further if desired. If not, the method development stops here. However, when still no good enantioseparation is achieved, which means that Rs < 1.5, each strategy suggests to switch to another separation technique or mode.

## 2. Capillary electrophoresis

Capillary electrophoresis is a technique that provides several advantages for chiral separations, such as high efficiency, a fast equilibration time and its ability to add various chiral selectors to the background electrolyte (BGE). Another important advantage of CE is the low consumption of reagents and sample during analysis which makes it possible to test new and/or expensive compounds and selectors.

The proposed separation strategy in CE is based on the use of three highly sulphated cyclodextrins (HSCDs), i.e. HS-α-CD, HS-β-CD and HS-γ-CD [14]. Its general set-up is shown in Fig. 1. This set-up was based on experimental and literature knowledge [7,13]. The selectors are screened at one set of conditions and in the sequence: (1) HS-γ-CD, (2) HS-β-CD and (3) HS-α-CD. For the experimental conditions of the screening step the phosphate buffer has a concentration of 50 mM and is set at a pH of 2.5. The cyclodextrin concentration is 5% (w/v). The applied electric field has a strength of 300 V/cm while the capillary temperature is 25 °C. The sequence of the selectors is based on their observed enantioselectivity. HS-γ-CD is the selector that shows enantioselectivity for most compounds, followed by the β and α forms [14].

Depending on the obtained results, several optimization steps are defined as shown in Fig. 1. The first one is the optimization of the Rs. This step (optimization 1) will be used when the compound is partly but not baseline separated at screening conditions. Depending on the analysis time at screening conditions, two different experimental designs are proposed. If the analysis time is less than 15 min, a $2^3$ full factorial design with one central point must be carried out. This means that three factors will be examined at two levels followed by one experiment defined at central conditions. The factors considered are the cyclodextrin concentration that varies from 2.5 over 6.25 to 10% (w/v), the pH of the BGE that is increased from 2.5 to 3.25 and 4 and the percentages of methanol in the BGE which will be added ranging from 0% over 7.5% to 15%.

If the analysis time is more than 15 min, the design is a 2 × 3 factorial design, where the concentration of CD and the pH of the BGE are the factors, the latter examined at two levels (2.5 and 4) while the former is examined at three levels (2.5%, 5% and 10% (w/v)).

For nearly baseline separated peaks, the strategy provides a second optimization possibility, which aims at improving the efficiency and the peak shape (optimization 2). This optimization also applies a $2^3$ full factorial design. The voltage (250 V/cm and 350 V/cm), temperature (15 °C and 25 °C) and ionic strength of the BGE (25 mM and 75 mM) are now the factors. For compounds that are baseline separated, a third optimization step (optimization 3) can be executed. The choice exists between a specific migration time optimization (performing a $2^3$ full factorial design) and a global optimization (performing a $2^{4-1}$

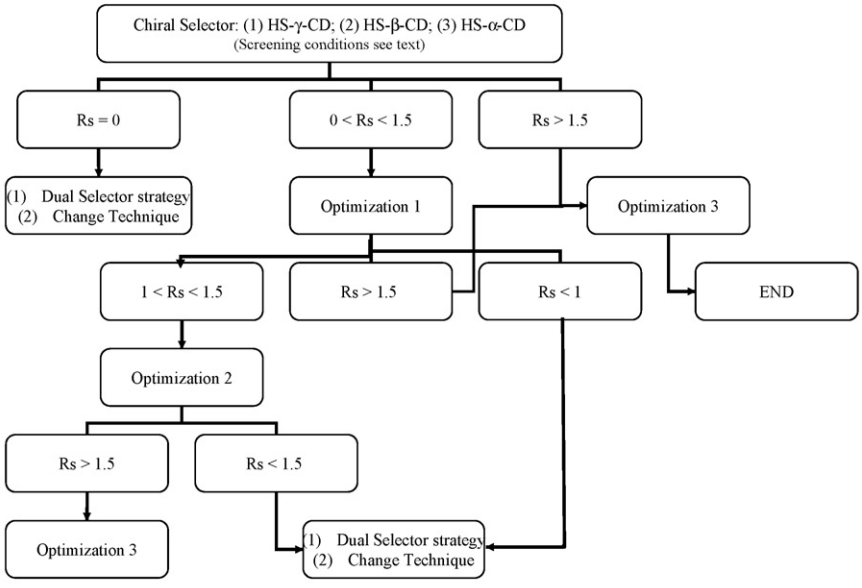


Fig. 1. General CE strategy.

fractional factorial design). In the $2^3$ design the voltage, temperature and CD-concentration are the varying factors. The levels of these factors are higher than the nominal levels at screening because their increasing will reduce migration time. The voltage is changed to 350 mV/cm and 400 mV/cm, the temperatures of the system are 25 °C and 35 °C while the CD concentrations are 7.5% and 10% (w/v). The conditions for global optimization are defined around the levels that were used previously. The responses optimized are plate number, asymmetry factor, migration time and resolution. Therefore the voltages considered are 250 mV/cm and 350 mV/cm, the temperature of the system is set at 15 °C and 25 °C, while the ionic strength of the BGE is changed to 25 mM and 75 mM, and the CD concentrations are 2.5% and 10% (w/v).

The CE strategy is not only applicable for the separation of racemic mixtures but was also demonstrated as being useful for the enantiomeric impurity determination [15]. The resolution is then preferably at least 3.0 instead of 1.5, due to the large difference in concentrations between active substance and enantiomeric impurity.

This CE strategy was applied on a test set of 40 compounds [7]. The strategy gave an enantioselectivity of 98% (39 compounds out of 40). A baseline separation was achieved for 90% of the analytes. After the first screening step 29 compounds were already baseline separated, while the optimization could separate another 7 new compounds [7].

When no enantioseparation is seen with any of the three HS-CDs, a dual cyclodextrins system can be tested, which combines a negatively charged HS-CD with a neutral CD [16]. Combining the two types of CDs may lead to better separations [17]. In this dual CD-part of the strategy, basic and non-basic compounds are treated differently, i.e. different concentrations of background electrolyte are used. The dual-CD-based strategy also has three optimization stages analogous to the HSCD-based strategy. For baseline separated compounds, no further optimization is necessary but a migration time (Mt) optimization can optionally be performed. For $0 < Rs \leq 1.5$, the resolution is optimized by varying the concentration of the neutral CD in the BGE at the levels 5 mM, 25 mM, 50 mM and 100 mM. When after applying the extended strategy (HSCD and dual CD systems) still no enantioseparation is achieved, it is recommended to use another technique to develop the separation [16].

## 3. High-performance liquid chromatography

The second technique for enantioseparation that will be discussed in this paper is HPLC. In HPLC, enantiomers can also be separated by adding chiral selectors to the mobile phase using a conventional achiral column as stationary phase. However, stationary phases with bonded or coated chiral selectors are more often used. Many types of chiral stationary phases exist but the polysaccharide-based CSPs have shown the broadest enantioselectivity so far [18,19]. A very useful property of these CSPs is the fact that they are developed for different HPLC modes such as normal- and reversed-phase liquid chromatography. The stationary phases developed for the latter mode can also be used in polar organic solvent chromatography (POSC) [12,13]. The POSC mode is gaining importance in the field of enantioseparations. Polar organic solvents (such as ethanol, methanol and acetonitrile) have different advantages. For instance the increasing solubility of some analytes, but also an alternative chiral recognition mechanism can occur when only polar organic solvents are used [20]. The stationary phases applied in NPLC on the other hand can also be used in supercritical fluid chromatography [11,13].

Literature shows that mainly derivatives of cellulose and amylose are used and that they are able to separate more than 80% of the drugs which are currently available on the market [21–23]. The stationary phases that are used in the below described strategies have a cellulose *tris* (3,5-dimethylphenylcarbamate)-, amylose *tris* (3,5-dimethylphenylcarbamate)-, cellulose *tris* (4-methylbenzoate)- and amylose *tris*-[(*S*)-α-methylbenzylcarbamate] as chiral selectors. These CSPs are better known under their commercial names Chiralcel® OD, Chiralpak® AD, Chiralcel® OJ and Chiralpak® AS, respectively. Each of the below discussed HPLC strategies applies UV detection at a wavelength of 220 nm.

### 3.1. Normal-phase liquid chromatography

In the normal-phase mode strategy the basic compounds are analyzed separately from the non-basic (see Fig. 2).

The reason is that the selectors are neutral and are most enantioselective when the analyzed compounds are also neutral. Therefore basic compounds are analyzed at high pH and the non-basic at low. The used CSPs are the normal-phase versions of the above mentioned polysaccharides. The first part of the strategy is a screening step which is based on two experimental designs. The screening of the basic compounds uses a $2^2$ full factorial design i.e. two factors are examined at two levels, requiring four experiments. The considered factors are the type of column, Chiralpak® AD and Chiralcel® OD, and the type of organic modifier (OM) in the hexane-based mobile phase, i.e. isopropanol (IPA) and ethanol (EtOH).

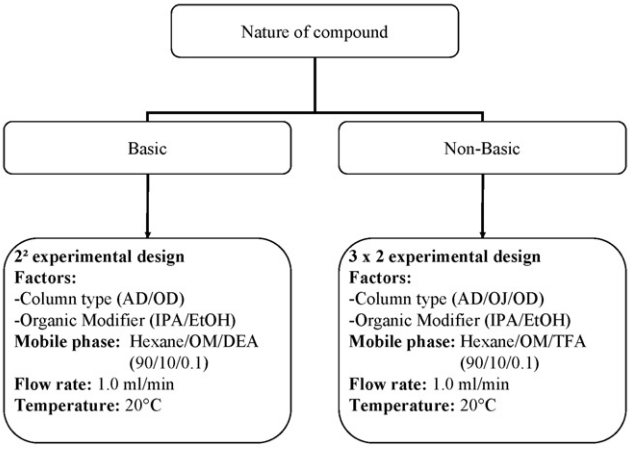


Fig. 2. NPLC screening strategy.

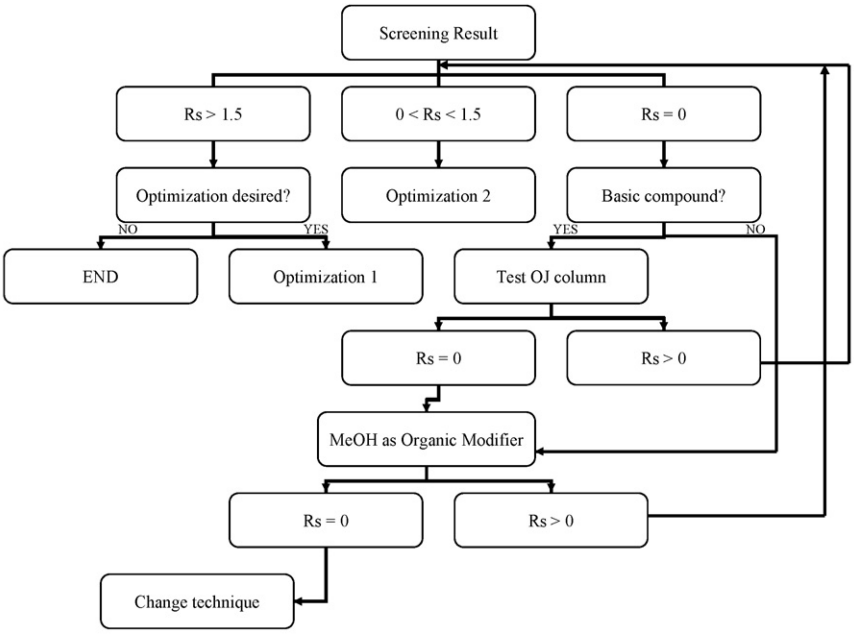


Fig. 3. NPLC optimization steps.

The screening of the non-basic compounds uses a 3 × 2 factorial design. This design includes two factors, one tested at three levels and the other at two, thus six experiments will be performed. The column type is now examined at three levels (Chiralpak® AD, Chiralcel® OJ and Chiralcel® OD) and the type of OM at two (IPA and EtOH). Other conditions such as temperature, mobile phase contents and flow rate are constant. The mobile phase also contains an additive which is diethylamine (DEA) for the basic compounds and trifluoroacetic acid (TFA) for the non-basic. It is known that the use of mobile phase additives can be essential to obtain good resolutions and to improve the column efficiency [21]. Here, they are also used to determine the pH.

After the screening, an optimization can be carried out depending on the results [10]. The results of the screening again can be divided into three categories (see Fig. 3). When immediately a baseline resolution is obtained, optimization of the peak shape and/or analysis time can be carried out (optimization 1). For the optimization of the peak shape, a 3 × 2 design is proposed where the percentage of additive is decreased and examined at three levels (0.025%, 0.050% and 0.075%) and the analysis temperature at two (15 °C and 25 °C) around the screening conditions. To optimize the analysis time, first the percentage of organic modifier is increased and when this is insufficient, a $2^2$ full factorial design is proposed where the temperature and the flow rate are varied. The temperature is increased (25 °C and 40 °C) while the flow rate depends on the chromatographic system but is anyway above 0.5 ml/min.

For compounds that are partly separated, optimization 2 is used to bring them to a baseline separation. This step starts with the calculation of the retention factor k from the experiment with the highest resolution in the screening. Depending on the result different solutions are proposed. When $k < 1$ or $k > 5$ the percentage organic modifier must be respectively decreased or increased until $1 < k < 5$. When no satisfying result is obtained another technique is recommended. When the wanted result ($1 < k < 5$) is reached, one reconsiders the Rs since changing the percentage OM has an influence on the selectivity. When $0 < Rs < 1.5$ a $2^2$ full factorial design is executed. In this design the concentration of the additive in the mobile phase is changed between 0.025% and 0.075% (v/v). The second factor is the temperature that changes between 5 °C and 15 °C. The responses considered are Rs and the number of the theoretical plates $N$. For compounds with $Rs > 1.5$ further optimization can be executed if wanted. Therefore optimization step 1 is performed without changing the percentage OM since this is already done at the beginning of optimization 2.

After the screening, the Rs of the compounds will be calculated. When the compounds with the highest Rs have a $k$ that is already $1 < k < 5$, the above mentioned $2^2$ full factorial design is executed. The experiment with the best compromise among the considered responses is then selected. If acceptable, the optimization stops at this point. If no experiment has acceptable results, it is recommended to change to another technique. For an elucidating overview of this strategy we refer to the original paper [10].

When no beginning of separation is achieved after screening, the basic compounds are tested with Chiralcel® OJ, a column that was not included in the screening. When $Rs > 0$ for the basic compounds tested on the OJ column, optimization 2 should be executed. However, when still no separation is obtained, methanol (MeOH) is tested as organic modifier first on the columns defined in the screening and when still no resolution is obtained, also on the OJ column. The use of methanol is evaluated, not only for the basic compounds but also for the non-basics. When after this step still no separation is obtained, one is recommended switch to another technique.

The strategy in NPLC mode was tested for 36 compounds of which 32 compounds showed enantioselectivity and 24 had a baseline resolution [8].

### 3.2. Reversed-phase liquid chromatography

The screening step in the RPLC-mode strategy is a $3 \times 2$ factorial design (Fig. 4). The factors are the column type (three levels) i.e. Chiralcel® OD-RH, Chiralcel® OJ-RH and Chiralpak® AD-RH, and the buffer type (two levels). The letter 'R' in RH refers to the reversed-phase types (and the 'H' refers to the particle size i.e. 5 μm) of this polysaccharide CSP's. The mobile phase is a mixture of buffer/ acetonitrile 60/40 (v/v). In general, as already mentioned above, enantiomers are better separated on the used columns when they are uncharged [9]. Therefore the addition of a chaotropic salt, potassium hexafluorophosphate ($KPF_6$), to the acidic phosphate buffer (pH 2) is recommended. The $PF_6^-$ ion will neutralize the basic compounds that are positively charged in acidic solutions, by formation of an ion-pair. This allows analyzing basic compounds under acidic conditions. The second buffer considered is a borate buffer at pH 9.

When no enantioselectivity ($Rs = 0$) is seen, the strategy proposes to change the organic modifier type. Acetonitrile (ACN) is replaced by ethanol, isopropanol and methanol in that sequence. The solvent strength is kept constant which means that the fraction varies with the modifier used. For each new modifier, the screening design is executed. When all modifiers are examined and still no separation is achieved, the strategy recommends to change the technique.

For compounds that have a beginning of separation ($0 < Rs < 1.5$), the optimization consists of a $2^2$ full factorial design where the fraction OM and the temperature are decreased at two levels. When the four experiments prescribed by the design still do not result in $Rs > 1.5$, one is recommended to switch to another technique. When the Rs is larger than 1.5, there is a possibility to optimize the retention factor (analysis time) by optimizing the concentration of the chaotropic salt.

The developed RPLC strategy was tested for eight compounds including basic, acidic, bifunctional and neutral analytes. After screening and optimisation, seven compounds were baseline separated [10].

### 3.3. Polar organic solvent chromatography

In this technique only polar organic solvents are used as the basis for the mobile phase, while the above reversed-phase columns are used. Additionally, also a Chiralpak® AS-RH column is used. The testing sequence of the columns is (1) AD-RH, (2) OD-RH, (3) AS-RH and (4) OJ-RH (see Fig. 5).

The first screening step includes a sequential screening on the four columns with two mobile phases, starting with an acetonitrile based mobile phase containing both 0.1% (v/v) DEA and 0.1% (v/v) TFA. The second mobile phase is a methanol-based solution with again 0.1% (v/v) DEA as well as 0.1% (v/v) TFA. The addition of both additives is preferred in this strategy over their individual use "because it allows good separations and minimizes memory effects in later separations" [12]. Since the screening is sequential, the analyst can stop at the moment where a $Rs > 1.5$ is reached. When after the first screening step $Rs = 0$, a second screening step is proposed where 5% IPA is added to the mobile phase. Experiments are executed using the columns in the same sequence. If this step does not result in a (beginning of) separation, the use of another technique is suggested.

When after this second screening step some separation is obtained, the same step is used as when $0 < Rs < 1.5$ after the first sequential screening. It recommends an addition of 5% alcohol

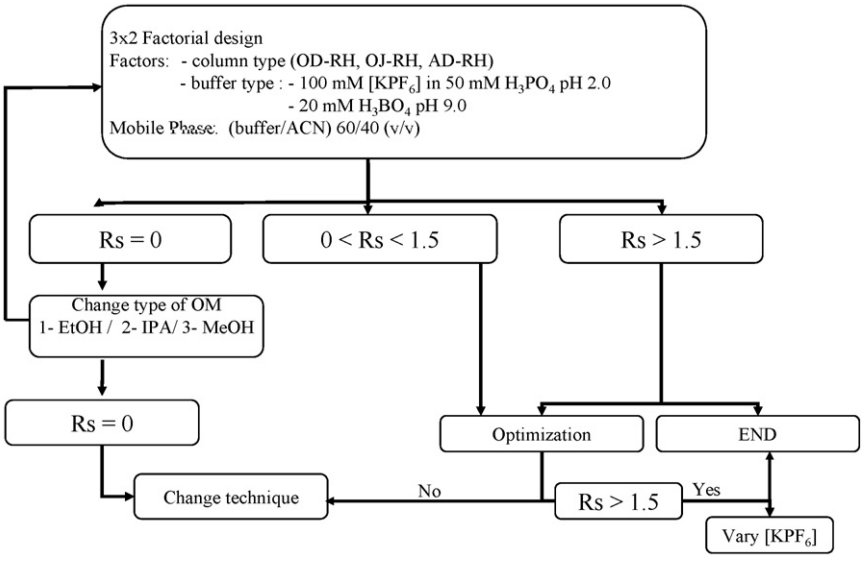


Fig. 4. RPLC strategy.

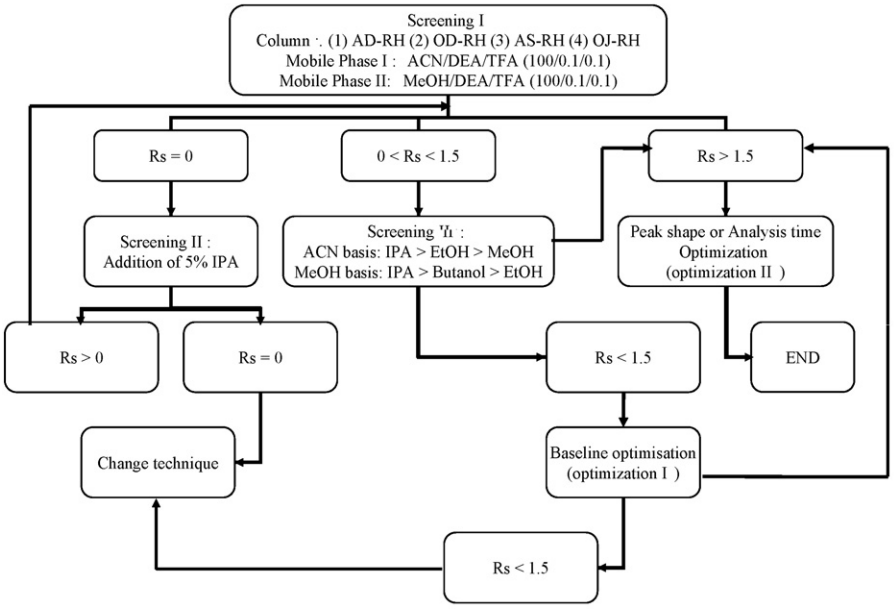


Fig. 5. POSC strategy.

to the mobile phase basis that gave the best result. The type and sequence of alcohols differ depending on the mobile phase basis. For the acetonitrile-based mobile phase, the proposed sequence is: isopropanol > ethanol > methanol, while for the methanol-based mobile phase isopropanol > butanol > ethanol is suggested [13]. If the result is still not satisfying (Rs < 1.5) an optimization (I) of the resolution is proposed by means of a $3 \times 2$ factorial design. The first factor to be changed is the fraction of organic modifier. The fraction depends on the mobile phase used i.e. for the ACN-based mobile phase the organic modifier will be 10%, 15% or 20%. For the MeOH-based mobile phase, it will vary from 15% over 50% to 85%. The second factor changed is the temperature which is reduced to 15 °C and 5 °C. When this step still results in Rs < 1.5, the change to another technique is suggested. If there is a baseline separation after this optimization, or after the first screening, an optional peak shape or an analysis time optimization (II) can be executed.

This strategy has been tested on 38 compounds. Enantioselectivity is observed for 35 compounds (=92%) of which 27 (=71%) were baseline separated [12].

## 4. Conclusion

Screening and optimization strategies for the separation of enantiomers in CE and different HPLC modes have been reviewed. The aim of each strategy was to enable fast chiral method development for any given compound. This also means that for specific compounds, the success rate for baseline separation could be improved when a more thorough optimization would be applied on the outcome of the strategy.

First, a screening is proposed and depending on the results optimization steps are suggested. When after the optimization step no enantioseparation is achieved with the applied technique, the strategy recommends switching to another. Both, screening and optimization steps are based on experimental and literature knowledge.

Only by keeping the strategies up-to-date, they can be used continuously in the future and global enantioselectivity rates will further increase. Because numerous new chiral selectors and CSPs are available or will be marketed, interesting ones, e.g. CSP with substituted polysaccharides, must be evaluated on their generic applicability and implemented into the existing strategies.

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