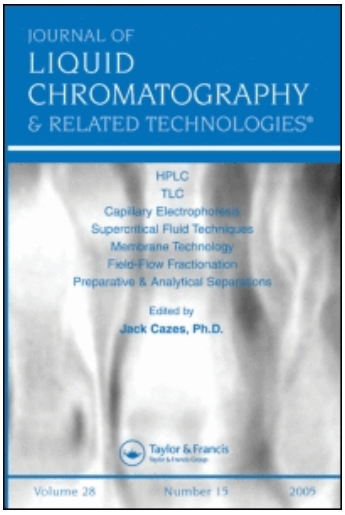


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# Effect of Some Parameters on Enantiomer Separation of Ephedrine, Methamphetamine and Selegiline Using HPLC with β-Cyclodextrin Stationary Phase

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# EFFECT OF SOME PARAMETERS ON ENANTIOMER SEPARATION OF EPHEDRINE, METHAMPHETAMINE AND SELEGILINE USING HPLC WITH β-CYCLODEXTRIN STATIONARY PHASE

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## ABSTRACT

The influence of different parameters (mobile phase composition - pH, organic solvent, salt nature and concentration; flow rate, injected amount and temperature) on enantiomeric separation of three pharmacologically important compounds (ephedrine, methamphetamine and selegiline) was studied using β-cyclodextrin stationary phase.

The evaluation of effect of these parameters allowed to optimize condition for optical purity determination. The following experimental conditions were chosen for separation of racemic mixture: stationary phase - ChiraDex, 5 μm; column - LiChroCart 250 × 4 mm I.D.; mobile phase - 500 mmol triethylamine/l with $H_2SO_4$ in water, pH = 3.5, flow-rate - 0.8 mL/min; detection - UV absorption at 206 nm; temperature - ambient.

The separation of minor (1%) enantiomer in excess of major one can be improved using flow-rate 0.2 mL/min and thermostated column (20°C for methamphetamine and selegiline, 5°C for ephedrine).

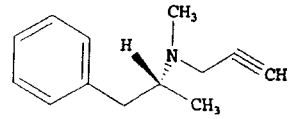
## INTRODUCTION

The importance of chirality in the natural world is well known. Many biologically important compounds show optical activity. The interest in the separations of chiral compounds has been growing rapidly over the past few years. The chromatographers in the different fields of work (pharmacy, agriculture, biotechnology, etc.) have to solve the problem of the enantiomer separation. For example, many pharmaceutical substances exhibit chirality and their enantiomers often have different pharmacological effects or different levels of activity. That is why the regulatory authorities require information about properties of individual enantiomers as well as about analytical techniques used for their separation. In the International Symposium on Purity Determination of Drugs (Stockholm, Sweden, 6 – 8 December 1993), Sven-Erik Hillver pointed out that "the presence of a non-wanted enantiomer could, in principle, be considered as any other impurity and hence, normal regulatory requirements and guidelines would be applicable."[1]

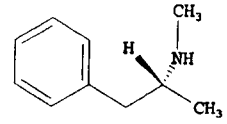
Among other separation techniques (CZE,[2] GC[3] etc.) the high performance liquid chromatography (HPLC) is also widely used for the optical isomer recognition.[4,5]

Different ways can be used in chiral separation by HPLC, such as the derivatization, the chiral additive in a mobile phase or the chiral stationary phase.

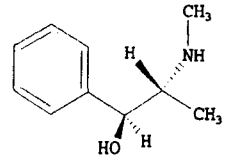
As the stationary phases, bonded cyclodextrins (native or derivatized) are often used.[4,5] In this work, native β-cyclodextrin stationary phase in the reversed phase chromatography mode has been tested for the enantiomer separation of three basic drugs - ephedrine (EP), methamphetamine (deoxyephedrine) (MAP) and selegiline (SEG) - *Deprenyl*, *Jumex*, (for structures see Fig. 1). These compounds are widely used or, especially in the case of MAP, abused for their pharmacological effects. They also represent different steps of pharmaceutical synthesis. The EP is a starting compound, MAP is an intermediate and SEG, (*R*)-(–) isomer, is a final product used as antidepressant and antiparkinsonian.




SEG




MAP




EP

**Figure 1**. Structures of studied drugs: SEG - selegiline, N-(1-phenylisopropyl)-N-methyl-N-propinylamine; MAP - methamphetamine, deoxyephedrine; EP - ephedrine, phenyl-2-methylamino-1-propanol.

The enantiomers of studied drugs were separated by HPLC after derivatization with chiral agents e.g. EP derivatized with (*S*)-( + )-1-(1-naphthyl)-ethyl isocyanate,[6] MAP derivatized with GITC or FLEC,[7,8] MAP and EP derivatized with GITC or FLEC,[9,10,11] where GITC is 2, 3, 4, 6-tetra-*O*-acetyl-β-D-glukopyranosyl isothiocyanate and FLEC is (+)- or (–)-enantiomer of 1-(9-fluorenyl)ethyl chloroformate.

The chiral stationary phases of Pirkle-type,[12] cellulose-type,[13,14] for MAP and cellulose-type[15] for MAP and EP were also used. The separation on cyclodextrin stationary phase for MAP was published.[16]

We successfully used a native β-cyclodextrin stationary phase for the separation of optical isomers of all named drugs. The influence of the different experimental parameters (pH, nature and concentration of salt, temperature etc.) on the separation was studied and evaluated in detail. Optimization of the enantiomer recognition has led to the method for the minor isomer determinations in the excess of the major one.

Concerning the practical application, we were looking for one mobile phase that would allow routine analyses of all three drugs with 1% level of minor isomer. It should be stated that for this purpose we did not search for an optimum of enantiomeric separation of individual compounds that can be, in general, different for each of them.

## EXPERIMENTAL



The chromatographic work was carried out by a liquid chromatograph Spectra Physics (pump SP 8700, UV/VIS detector SP 8440, all Spectra-Physics, San Jose, CA, USA). UV absorption chromatograms were recorded at 206 nm and 258 nm respectively. The chromatographic station CSW version 1.0 (DataApex, Prague, Czech Republic) was used for chromatogram acquisition and handling. The samples were injected by a 10 μL syringe (Hamilton, Reno, NV, USA) in a manual 7125 injector equipped with a 10 μL loop (Rheodyne, Cotati, CA, USA).

The separations were performed using a 250 × 4 mm I.D. ChiraDex 5 μm LiChroCart column (E. Merck, Darmstadt, F. R. Germany). The temperature of the column was controlled with the precision ± 0.1°C using a glass water jacket and a laboratory water thermostat equipped with a freon cooler. The flow rate was changed among 0.2 - 1.0 mL/min (see results and discussion).

The mobile phases were prepared by volume by volume mixing of components. As these components, HPLC grade acetonitrile and tetrahydrofuran (E. Merck, Darmstadt, F. R. Germany), UV grade methanol (Lachema, Brno, Czech Republic) and salt solutions were used. The salt solutions were prepared by dissolution of adequate amounts of salt or triethylamine (TEA) in redistilled water and pH was set up by corresponding acid concentrated - $CH_3COOH$, HCOOH, $H_3PO_4$ or diluted - $H2SO_4$, $HNO_3$, $HClO_4$ (20% or 5% (v/v) solutions). The abbreviation TEAS is used for combination of TEA with $H_2SO_4$ and mentioned concentration is related to TEA. All chemicals used for this purpose were of analytical grade.

**Table 1**

**Chromatographic Parameters (See Text) Versus pH, Salt Cation ($M^+$), Salt Concentration (c) and Temperature (T) for Ephedrine (EP), Methamphetamine (MAP) and Selegiline (SEG)**

| Studied Parameter | | $k_{c,rel}$ | | | $\alpha_{c,rel}$ | | | $S_{rel}$ | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | EP | MAP | SEG | EP | MAP | SEG | EP | MAP | SEG |
| pH | 3.5 | 0.59 | 0.80 | 0.87 | 0.73 | 1.00 | 0.83 | 0.53 | 1.00 | 0.69 |
| | 4.5 | 0.65 | 0.84 | 0.92 | 0.71 | 0.90 | 0.79 | 0.58 | 0.95 | 0.91 |
| | 5.5 | 0.77 | 0.92 | 1.00 | 0.69 | 0.78 | 0.67 | 0.66 | 0.90 | 0.84 |
| $M^+$ | $Na^+$ | 0.87 | 0197 | 1.00 | 0.76 | 0.67 | 0.64 | 0.82 | 0.80 | 0.79 |
| | $NH_4^+$ | 0.86 | 0.96 | 0.99 | 0.77 | 0.71 | 0.67 | 0.82 | 0.84 | 0.81 |
| | $TEA^+$ | 0.73 | 0.86 | 0.90 | 1.00 | 0.94 | 0.87 | 0.90 | 1.00 | 0.97 |
| C (nmol/L) | 50 | 0.81 | 0.96 | 1.00 | 0.76 | 0.80 | 0.82 | 0.71 | 0.89 | 0.95 |
| | 100 | 0.82 | 0.96 | 1.00 | 0.81 | 0.83 | 0.84 | 0.76 | 0.93 | 0.98 |
| | 150 | 0.82 | 0.96 | 1.00 | 0.82 | 0.85 | 0.84 | 0.77 | 0.95 | 0.96 |
| | 250 | 0.82 | 0.96 | 1.00 | 0.87 | 0.87 | 0.85 | 0.82 | 0.97 | 0.99 |
| | 500 | 0.80 | 0.95 | 0.99 | 0.95 | 0.90 | 0.84 | 0.89 | 0.99 | 0.97 |
| | 750 | 0.80 | 0.94 | 0.99 | 1.00 | 0.91 | 0.82 | 0.93 | 1.00 | 0.95 |
| T (°C) | 5 | 0.83 | 0.95 | 1.00 | 1.00 | 0.84 | 0.81 | 1.00 | 0.97 | 0.98 |
| | 10 | 0.81 | 0.95 | 0.99 | 0.93 | 0.81 | 0.77 | 0.91 | 0.92 | 0.92 |
| | 15 | 0.80 | 0.94 | 0.98 | 0.87 | 0.78 | 0.74 | 0.84 | 0.87 | 0.87 |
| | 20 | 0.78 | 0.93 | 0.97 | 0.82 | 0.75 | 0.71 | 0.76 | 0.84 | 0.82 |
| | 25 | 0.76 | 0.92 | 0.96 | 0.76 | 0.72 | 0.68 | 0.69 | 0.79 | 0.77 |
| | 30 | 0.75 | 0.90 | 0.95 | 0.71 | 0.70 | 0.65 | 0.62 | 0.75 | 0.73 |

Stationary Phase: ChiraDex, 5µm. Column: LiChroCart 250 x 4 mm I.D. Mobile phase: for pH - 500 mmol TEA/1 with $H_2PO_4$ in water, for $M^+$ - 500 mmol cation/1 with $H_2PO_4$, pH = 3.5, for c - TEA with $H_2SO_4$, pH = 3.5 and for T - 500 mmol TEA/1 with $H_2SO_4$, pH = 3.5; flow-rate 0.8 mL/min. Detection: UV absorption at 206 nm. Injecton: 10 µL, 0.1 mg each enantiomer/mL water. Temperature: for pH, $M^+$ and c ambient.

Hold-up volumes were determined by triplicate injections of water (10 mL) with the detection wavelength 200 nm, mobile phase methanol : water = 40 : 60 and flow-rate 0.8 mL/min. Mean values of two retention times were used for calculation of retention factors.

The hydrochlorides of all studied enantiomers - (*1S,2R*)-(+)-EP, (*1R, 2S*)-(–)-EP, (*R*)-(–)-MAP, (*S*)-(+)-MAP, (*R*)-(–)-SEG and (*S*)-(+)-SEG) - were gifts of Farmakon, Olomouc, Czech Republic. The contents of the minor enantiomer in major one was undetectable using evaluated methods. The concentration of each enantiomer in the stock solution was 2 mg/mL of redistilled water.

## RESULTS AND DISCUSSION

Starting information on the retention of the studied substances was obtained on the base of some preliminary experiments with mobile phases methanol - phosphate buffer (50 mM-$Na_2P\ O_4$, pH 3.5 or 7.5 with $H_3PO_4$). It was found out that for pH 3.5 the compounds can be eluted from the column only with the buffer solution without any organic solvents. For the evaluation of separation quality we used separation factor S (1),[17] resolution $R_s$ (2)[18] and relative values $k_{c,sel}$ (6), $a_{c,sel}$ (7) that characterize the contribution of capacity and selectivity respectively to the separation of studied enantiomers.

$$S = (k_2 - k_1)/(k_2 + k_1 + 2) \qquad (1)$$

where $k_1$ and $k_2$ is a capacity factor of compound with lower and higher retention respectively.

$$R_S = 1/4 \cdot n^{1/2} \cdot (a_{1,2} - 1)/a_{1,2} \cdot k_2/(k_2 + 1) \qquad (2)$$

where $a_{1,2}$ is a relative retention and n is a number of theoretical plates. In this work, the resolution was used for the good separated peaks ($R_s > 1.1$), in other cases the total quality of separation was estimated with the use of separation factor S and with the consideration of the peak tailing. The other applied parameters were

$$k_c = k_2/(k_2 + 1) \qquad (3)$$

$$\alpha_c = (\alpha_{1,2} - 1)/\alpha_{1,2} \qquad (4)$$

where $k_c$ and $\alpha_c$ means respectively the contribution of capacity and selectivity to the resolution.

$$k_{c,rel} = k_c / k_{c,max} \quad (5)$$

$$\alpha_{c,rel} = \alpha_c / \alpha_{c,max} \quad (6)$$

$$S_{rel} = S / S_{max} \quad (7)$$

where $k_{c,max}$, $\alpha_{c,max}$ and $S_{max}$ are the maximum values of $k_c$, $\alpha_c$ and S from the evaluated data set.

**The Effect of pH and Organic Solvent**

We evaluated the effect of pH in the range of column stability (from 3.0 to 7.5). Table 1 shows the impact of pH change from 3.5 to 5.5 where we used a mobile phase without organic solvent. As can be predicted, $k_{c,sel}$ is increasing (most for EP). The studied drugs are basic compounds and owing to protonization the retention is lower for lower pH. Hydroxyl group of EP decreases and propargyl group of SEG increases the retention of these compounds in comparison to MAP. For all experimental pH values, the drugs are eluted in the order of EP, MAP and SEG.

The selectivity is improving (higher $\alpha_{c,sel}$, Table 1) with pH diminishing but only slightly for EP. The combined effect of capacity and selectivity appears in S. At pH 5.5 $S_{rel}$ shows the deterioration of separation for SEG and MAP but the improvement for EP. It means that for the first two drugs the decrease of selectivity is not compensated by increase of capacity. For EP the higher capacity factor is the cause of the better separation. Because the anion optimization (see below) led to the increase of capacity (the critical parameter in EP separation), it was possible to apply pH = 3.5 as optimum for all drugs. We can also expect the better robustness of method in pH = 3.5. The small change in acidity does not mean so high change of the retention as it could occur in pH = 5.5 (impact of equilibrium of protonized and non-protonized form).

The effect of pH on the separation near the optimum with the optimal salt nature and concentration (TEAS, 500 mmol/L) is small. The resolution is relatively good for all enantiomer couples (1.33 - 1.38 - 1.33 for EP, 1.46 - 1.50 - 1.48 for SEG and 1.55 - 1.57 - 1.56 for MAP in order of pH - 3.0, 3.5, 4.0).

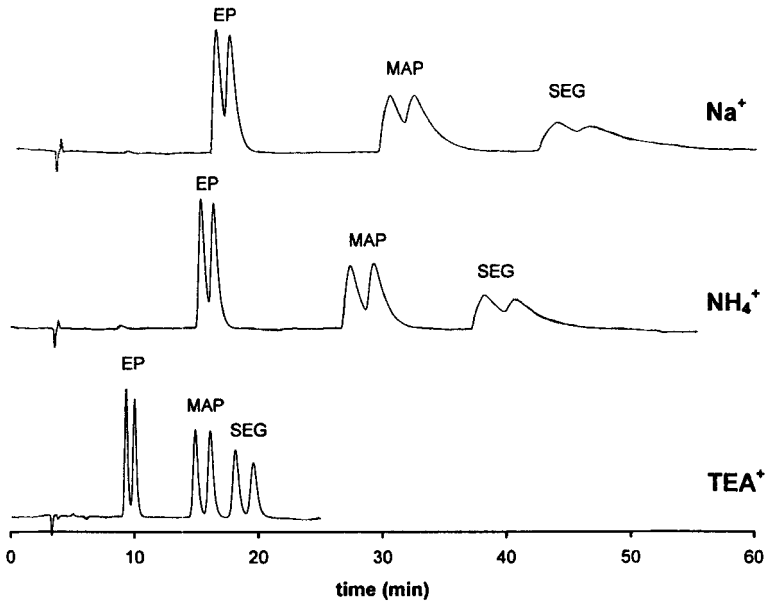



**Figure 2**. Chromatogram of ephedrine (EP), methamphetamine (MAP) and selegiline (SEG) for different cations of salt in a mobile phase. Stationary phase: ChiraDex, 5μm. Column: LiChroCart 250 x 4 mm I.D. Mobile phase: 500 mmol cation/l with $H_2SO_4$, pH = 3.5; flow-rate 0.8 mL/min. Detection: UV absorption at 3\206 nm. Injection: 10μL, 0.1 mg each enantiomer/mL water. Temperature: ambient.



To keep retention in approximately the same level for different pH values (3.5, 4.5, 5.5, 6.5 and 7.5) the methanol was added to the mobile phase in appropriate amount (in dependence on pH). These experiments led to the same conclusion about pH optimum as described above.

For pH = 3.5 and optimal salt nature as well as concentration, the effect of organic solvents (methanol, acetonitrile, tetrahydrofuran) was evaluated. In all cases the retention, but also the separation, was decreasing. That is why the organic solvents were not used in the mobile phases and this parameter was not optimized in detail.

We can conclude that protonized forms of drugs show a good selectivity of separation from following the differences in the formation of diastereomeric inclusion complexes, and a lower capacity in comparison with non-protonized forms, owing to the lower stability of the charged molecule complexes.

**The Effect of theNature of the Salt**

For pH optimum the impact of three cations ($Na^+$, $NH_4^+$ and $TEA^+$) in combination with $PO_4^{3-}$ or $SO_4^{2-}$ respectively was studied. In the case of $PO_4^{3-}$, the choice of optimal cation ($TEA^+$) was made on the base of peak shape. The use of $TEA^+$ allows one to attain the better peak shapes especially for SEG and MAP. The differences in $k_{c,sel}$, $\alpha_{c,rel}$ and $S_{rel}$ are more evident for combination of cations with $SO_4^{2-}$ that was found as the best for our purposes (see below).

The parameters are changing in the order $Na^+$ - $NH_4^+$ - $TEA^+$, $k_{c,sel}$ is decreasing, $\alpha_{c,sel}$ and $S_{rel}$ are increasing (Table 1). Again (as for pH), the decrease of capacity contribution for EP is higher in comparison with other two drugs, but in this case the increasing selectivity compensates a loss in $k_{c,sel}$. $TEA^+$ offers the best separation in the shortest time in comparison with the other two tested cations, where peak tailing also contributes to the deterioration of the enantiomer recognition (Fig. 2).

We can suppose that basic compounds separated as cations compete with cations of salt in the cyclodextrin cavity occupation as well as in the interaction with the hydroxyl groups. This competition leads to the decrease of drug retention with the cation change ($Na^+$ - $NH_4^+$ - $TEA^+$) as well as to the peak shape improvement in the same order. In the same sense the "salting out" effect (the highest for $Na^+$, the lowest for $TEA^+$) acts on the retention.

In the next step the nature of anion was optimized in combination with $TEA^+$. Its concentration was 150 mmol/L for univalent and 100 mmol/L for bivalent anions, to keep approximately constant ionic strength.

The anions can be arranged in the succession by the increasing drug retention - $ClO_4^- < CH_3COO^- < NO_3^- < HCOO^- < PO_4^{3-} < SO_4^{2-}$. In this order $k_{c,sel}$ is increasing for all studied compounds (Fig. 3) owing to the increasing hydratation of anions. The graph of $\alpha_{c,rel}$ is slightly more complicated. The best separation for $SO_4^{2-}$ was found out. For this anion in comparison with the others, the EP shows the highest $k_{c,rel}$ as well as $\alpha_{c,rel}$, MAP and SEG show highest $k_{c,rel}$ but not $\alpha_{c,rel}$. However, the capacity increase has higher impact on the separation than decrease of selectivity, as seen from $S_{rel}$ graphs.

The studied compounds have the absorption maximum around 206 nm that is by order higher than side maximum at 258 nm. It was used in anion optimization experiments together with higher injected amount.

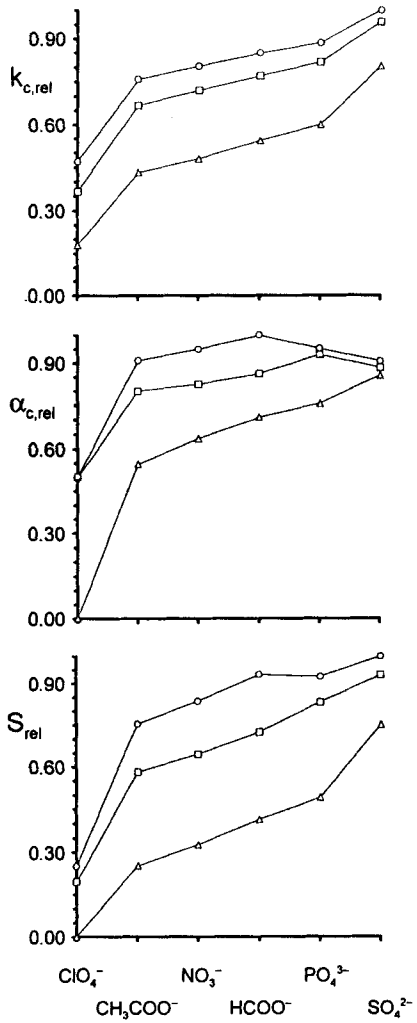



**Figure 3**. Chromatographic parameters (see text) versus anion of salt for ephedrine (Δ).methamphetamine (□) and selegiline (○). Mobile phase: 500 mmol TEA/L with acid of correpsonding anion, pH = 3.5. Detection: UV absorption at 258 nm. Other conditions as in Figure 2.

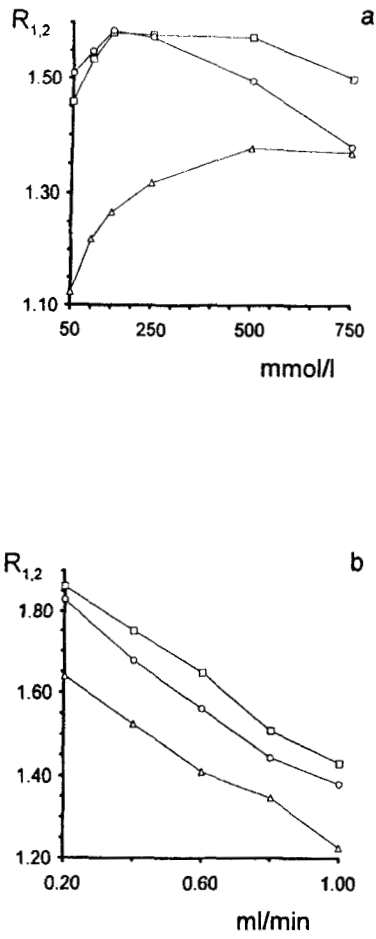



**Figure 4**. Resolution versus salt concentration (4a) and versus mobile phase flow-rate (4b) for ephedrine (Δ), methamphetamine (□) and selegiline (○). Mobile phase: 4a - TEA with $H_2SO_4$, pH = 3.5. Other conditions as in Figure 2.

Some studied anions ($ClO_4^-$, $CH_3COO^-$, $NO_3^-$, $HCOO^-$) in used concentration did not allow the detection at 206 nm due to too high mobile phase absorptivity. For the enantiomeric separation the injected amount of drugs is very important (see below). The detection at 206 nm allows its decrease.

Anion $SO_4^{2-}$ shows the good behaviour in both cases (detection as well as chromatographic separation) and is the most convenient one. It was used in mobile phases for verification of above optimized parameters (pH, cations) as well as in the following optimization steps.

**The Salt Concentration**

The dependencies of the $k_{c,rel}$, $\alpha_{c,rel}$, $S_{rel}$ and $R_s$ on the salt concentration are shown in the Table 1 and in Fig. 4a, respectively. The contribution of the capacity change to the resolution is not as important as the change of selectivity, especially for EP. The resolution that is also dependent on efficiency, after starting improvement, becomes smaller for all three compounds. The difference is in the size and starting concentration of decline (Fig. 4a).



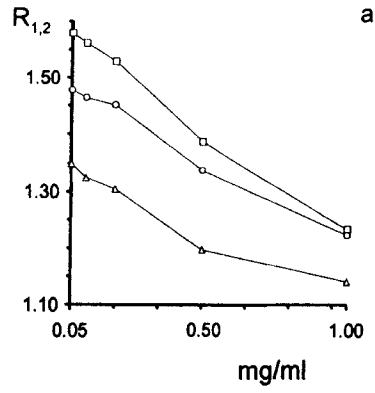
For the next experiments the $TEA^+$ concentration 500 mmol/L was chosen (optimum for EP). The optimum for SEG and MAP lies around 150 mmol/L. However in both cases the resolution at 500 mmol/l is higher in comparison to EP and at the same time close to 1.5 (1.57 and 1.50 for MAP and SEG respectively).

The concentration effect on capacity can be explained by cation and anion contribution. The capacity becomes higher with the increase of $SO_4^{2-}$ concentration but lower with the increase of $TEA^+$ concentration. The result is a small change in the capacity.

**The Effect of Mobile Phase Flow-Rate**

The flow-rate impact on $k_{c,rel}$, $\alpha_{c,rel}$ and $S_{rel}$ is minor and the change of these parameters is more probably due to the reproducibility of measurements. The resolution is then effected by the change of efficiency. For all compounds the resolution of enantiomers is decreasing with higher flow rate (usual trend in HPLC) (Fig. 4b).

The increase of peak areas (typical for concentration detectors) and peak heights (increase of efficiency) with the flow-rate decrease contributes to the detection improvement. On the other side, in the same direction, the analysis time is increasing.



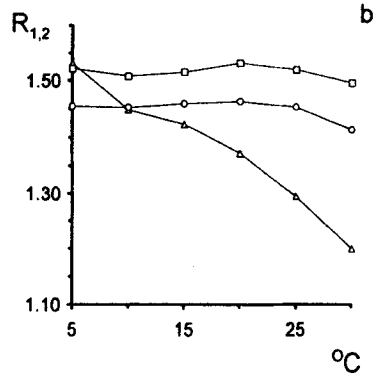






**Figure 5**. Resolution versus racemate concentration in injected solution (5a) and versus temperature (5b) for ephedrine (Δ), methamphetamine (□) and selegiline (○). Mobile phase: 500 mmol TEA/l with $H_2SO_4$, pH = 3.5. Other conditions as in Figure 2.

## The Effect of Injected Amount

The possibility to use lower detection wavelength (more sensitive detection) led to the test of injected amount impact on the resolution. Only neglected changes of $k_{c,rel}$, $\alpha_{c,rel}$ and $S_{rel}$ in the whole studied range were

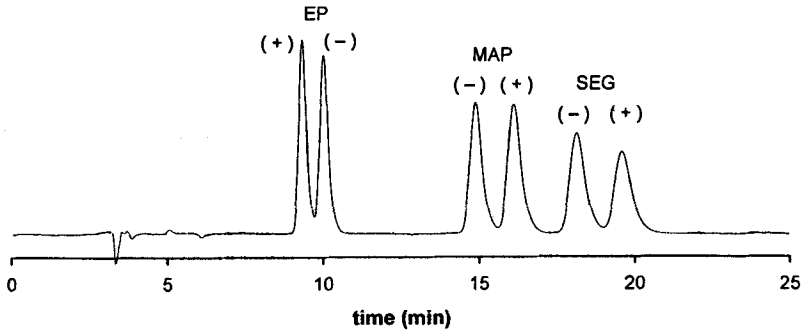



**Figure 6**. Separation of racemic mixtures of ephedrine (EP), methamphetamine (MAP) and selegiline (SEG). Mobile phase: 500 mmol TEA/l with $H_2SO_4$, pH = 3.5. Other conditions as in Figure 2.



observed. The change of resolution is more important (Fig. 5a). We can conclude that the change of number of theoretical plates (decrease of peak tailing) is the main factor contributing to the resolution improvement with the decrease of injected amount. The highest amount (for good detection) with a satisfactory resolution i.e. 0.2 mg racemic mixture/mL was selected as optimal. The importance of sensitive detection (see above discussion about the anion optimization) that allows diminishing of injected amount is evident.

The discussed effect can be explained by the nonlinearity of separation isotherm in the studied concentration range. With the injected amount decrease, we move to or closer to, the linear part of isotherm. It means the peak shape improvement, the decrease of peak tailing.

## The Effect of Temperature

The effect of temperature was studied in the range 5 - 30°C. With decreasing temperature, we can see the increase of $k_{c,rel}$, $\alpha_{c,rel}$ and of course $S_{rel}$ for all studied drugs (the highest for EP)(Table 1). The lower temperature is more convenient for the formation of the inclusion complexes.

Increase of complex stabilities (capacity factor increase) but also increase of difference in complex stabilities of the enantiomeric pairs (selectivity increase) is evident.

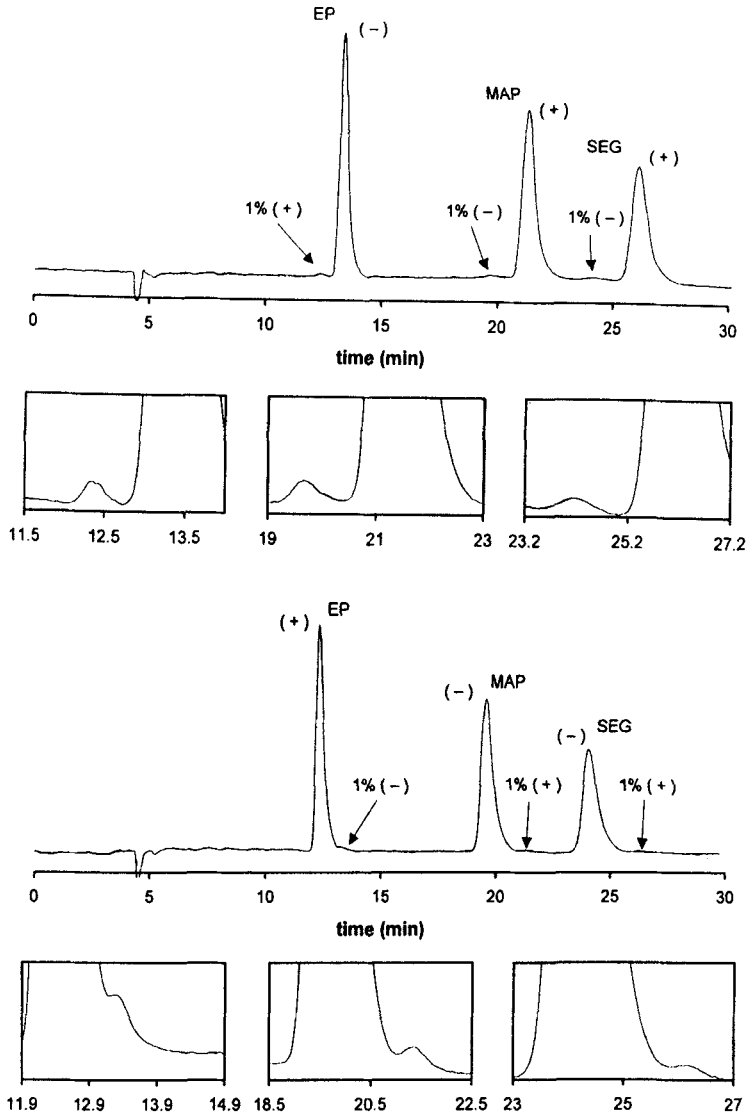



**Figure 7.** Separation of minor isomers of ephedrine (EP), methamphetamine (MAP) and selegiline (SEG). Mobile phase 500 mmol TEA/l with $H_2SO_4$, pH = 3.5; flow-rate 0.6 mL/min. Injection: 10μL, injected solution - 0.1 mg major and 0.001 mg minor enantiomer/mL water. Other conditions as in Figure 2.

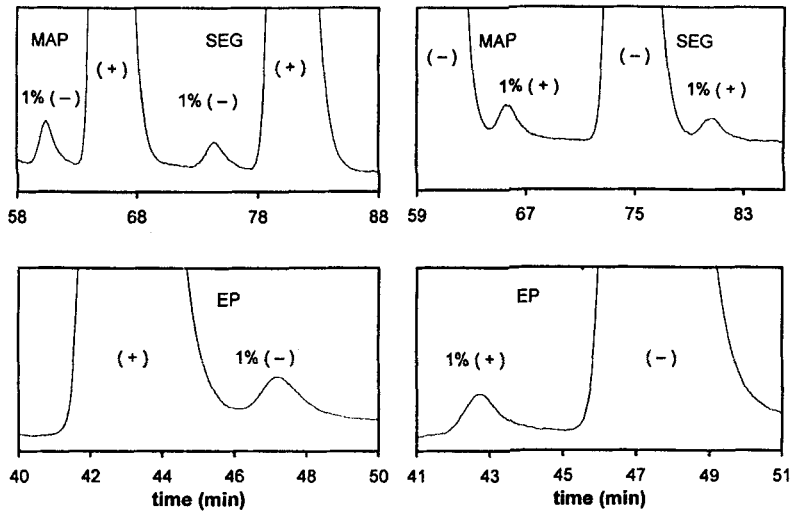



**Figure 8.** Separation of minor isomers of ephedrine (EP), methamphetamine (MAP) and selegiline (SEG). Mobile phase: 500 mmol TEA/l with $H_2SO_4$, pH = 3.5; flow-rate 0.2 mL/min. Temperature: 20°C for MAP an SEG, 5°C for EP. Injection: 10μL, injected solution -0.1 mg major and 0.001 mg minor enantiomer/mL water. Other conditions as in Figure 2.



The resolution is increasing in whole studied temperature range for EP but not for MAP and SEG (Fig. 5b). The difference between $S_{rel}$ and $R_s$ dependencies can be explained by the decrease of efficiency at lower temperatures (the decrease of diffusion, increase of mobile phase viscosity). From a practical point of view, the increase of working pressure for the same flow-rate in lower temperatures should not be forgotten.

## Application of Optimized Method

The optimization procedure led to the method that allows analysis of racemic mixture (Fig. 6) as well as the determination of 1% of a minor enantiomer (10 ng injected) in the excess of a major one (Fig. 7). Other improvements can be reached by decreasing flow-rate and temperature (Fig. 8), of course with the longer analysis time.

The column was used more than 100 hours. During this time the efficiency and peak symmetry became worse but enantiomer separation was still acceptable. The column washing, after daily work, as well as the use of a precolumn is highly recommended.

The method validation for the analysis of pharmaceutical substance (SEG) is in progress.

## CONCLUSION

The evaluation of the effect of studied parameters (pH and composition of mobile phase, injected amount etc.) allowed one to optimize the chiral separation on cyclodextrin stationary phase . Each parameter contributes to the final result. Some starting knowledge (about retention, detection, injected amount) is very useful, e.g. very high injected amount can make the separation impossible but also can lead to the omission of influence of other optimized parameters (some improvement of separation can not be recognized). The described approach can also be applied to the solution of a chiral separation of other compounds using b-cyclodextrin stationary phase.

An optimized method allows optical purity determination of all studied compounds using the same mobile phase that is useful for the routine control of production process. The presence of minor enantiomer below 1% can be found out.



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