Journal of Chromatography A, 1218 (2011) 5157–5165

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

# The influence of the memory effect on preparative separations using the amylose tris(3,5-dimethylphenylcarbamate) stationary phase

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ARTICLE INFO

*Article history:*
Received 24 March 2011
Received in revised form 21 May 2011
Accepted 23 May 2011
Available online 1 June 2011

*Keywords:*
CHIRALPAK AD
Memory effect
Ethanesulfonic acid
Reproducibility
Preparative chromatography

ABSTRACT

Acid/base mobile phase modifiers affect enantioseparations in ways that are not yet understood for the lack of systematic studies, which makes the scale-up of preparative separations difficult to predict. Shifts of the selectivity of certain pairs of enantiomers upon exposure of the column to these modifiers is amply documented. Furthermore, once the modifier has been removed from the mobile phase, the improved selectivity remains, this phenomenon has been named the memory effect. We selected four enantiomeric pairs for a systematic study of this memory effect. The selectivity of 4-chlorophenylalanine ethyl ester (4CPEE) improves after a solution of ethanesulfonic acid (ESA) is percolated through the column. The selectivity of propranolol HCl and Tröger's base increases after a solution of diiospropylethylamine is percolated through the column. The selectivity of these three pairs of enantiomers is inversely affected by percolation of the opposite acid/base solution. Each of these four compounds reached an equilibrium concentration that maintained the separation of the enantiomeric pairs. In contrast, the selectivity of *trans*-stilbene oxide (TSO) is not affected by either acid/base modifier. Preparative separations can be used to detect changes in the active surface of the chiral polymer stationary phase by measuring the change in selectivity and resolution when modifiers are used. Preparative method development was carried out on analytical columns and scale-up to 1 cm ID columns were performed in this study.



## 1. Introduction

Chiral purification of the enantiomers of compounds that are used in the preparation of pharmaceuticals, food products, or even pesticides has grown in demand as better separation methods were developed [1]. Research on these enantiomers suggests that only one of them is of importance in each specific case [2], which has lead to higher demands for one of the enantiomers to be collected in highly enriched amounts. In the case of pharmaceuticals, the FDA requires that, if both enantiomers are used in a formulation, both be studied fully and the effects of both be documented [3]. Numerous methods exist to carry out these purifications, among which crystallization, asymmetric synthesis, and chromatographic separation are the most common [4].

Asymmetric synthesis requires that pure enantiomers be synthesized to ensure that the analytical purification tests are accurate. As a result, crystallization and chromatographic purifications of racemic mixtures are usually done early in the production process of drugs at scales large enough to complete the test required to fulfil the FDA requirements. Since many analytical methods involve chromatographic techniques, chromatographic preparative methods to purify enantiomers are used to produce the amount of the pure enantiomer required. Stationary phases based on amylose and cellulose derivatives of tris(3,5-dimethylphenylcarbamate) have been used successfully to carry out large scale purifications of pharmaceuticals (10s of grams to kilograms) that satisfy the FDA requirements [5]. The amylose derivative (CHIRALPAK AD, Diacel Industries, Osaka, Japan) is used for the manufacturing of a number of the pharmaceuticals on the market today [6–8]. Even though this stationary phase is used in numerous steps of the drug testing process, one of its weaknesses has caused difficulty in the scaling of preparative chromatographic methods. This weakness is the acid or base memory effect [9,10]. The memory effect is most pronounced when non-polar mobile phases are used. The memory effect of CHIRALPAK AD has been documented for a number of racemic mixtures [11,12].

In this study the separation of two racemic mixtures – 4-chlorophenylalanine ethyl ester (4CPEE) and propranolol – which have not been purified in large quantities using chromatographic techniques is described. Both the 4CPEE and the propranolol are influenced by the presence of the memory effect. Two additional racemic mixtures: *trans*-stilbene oxide (TSO) and Tröger's base; have been separated numerous times on the CHIRALPAK AD [13,14]. Any changes in the separation capacity of these two racemic mixtures which are due to the memory effect have not been documented. Previously it was shown that even though the

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doi:10.1016/j.chroma.2011.05.084

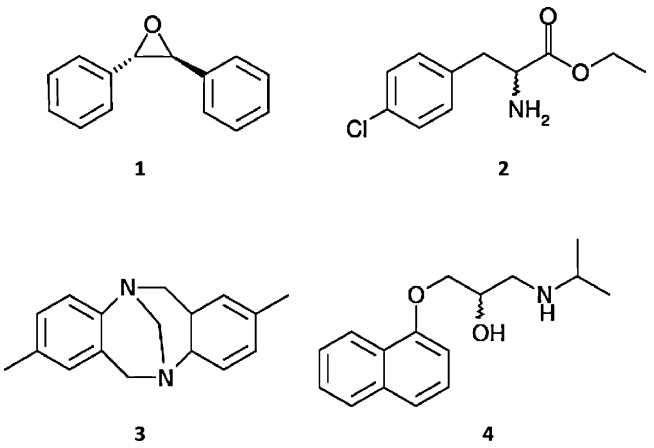


**Fig. 1.** Structure of chiral racemic mixtures – 1: *trans*-stilbene oxide; 2: 4-chlorophenylalanine ethyl ester; 3: Tröger's Base; 4: propranolol.

stationary phase has been exposed to both acid and base solutions, the columns can be used for analytical modelling [12]. The chemical structures for all four racemic mixtures can be seen in Fig. 1.

Tröger's base and TSO are considered to be standards in determining the CHIRALPAK AD columns efficiency and the overall quality of this packing material [15,16]. Used as the standards, these racemic mixtures can determine the quality and number of active sites available at the preparative level for a specific stationary phase. The separation of these two racemic mixtures could indicate if the stationary phase has been damaged by exposure to ethanesulfonic acid or N,N-diisopropylethylamine. The preparative separation of these two racemic mixtures should also indicate if the neutral active sites are involved in the memory effect.

The separation of the 4CPEE enantiomers is affected by the influence of an acidic modifier introduced into the mobile phase prior to the injection of analytical amounts of these enantiomers (see Fig. 2a). Without the use of an acidic mobile phase modifier both enantiomers elute as one peak (see Fig. 2a, solid line). The lack of selectivity change after removing the acid mobile phase modifier from the column (see Fig. 2a, broken line) is called the acid memory effect (AME). Conversely, the separation of the enantiomers of propranolol is affected by the addition of a basic modifier to the mobile phase prior to the analysis of these enantiomers (see Fig. 2b, solid line). The lack of change in selectivity after removing the basic mobile phase modifier from the column (see Fig. 2b, broken line) is called the base memory effect (BME). At the preparative scale, the separations of these two enantiomers should provide information on the stability of the active sites influenced by the memory effect. When developing a separation method on an analytical column and scaling it up to a preparative column, the influence of the memory effect on the preparative separation achieved can be observed depending on the quality of the separation method.

## 2. Experimental

### 2.1. Chemicals

The mobile phase used in the following experiments consisted of hexanes obtained from Fisher Scientific (Pittsburgh, PA, USA) and manufactured by JT Baker (Phillipsburg, NJ, USA) this product contains more than 95% n-hexane, with less than 2% methylcyclopentane and small amounts of branched hexanes. The alcohol modifier of the hexanes was ACS reagent grade alcohol obtained by Fisher Scientific. ACS reagent grade alcohol contains 90% ethyl alcohol, 5% isopropyl alcohol, and 5% methyl alcohol. Chemicals obtained from Sigma–Aldrich (St. Louis, MO, USA) included ethanesulfonic acid (ESA) – 95%, 4-chlorophenylalanine ethyl ester – 97% (4CPEE), 1,3,5-tri-*tert*-butylbenzene – 97% (TTBB) used as a column void marker, propranolol hydrochloride – 99%, and Tröger's base, and the *trans*-stilbene oxide – 97% was obtained from Acros Organics. The N,N-Diisopropylethylamine was obtained from Alfa Aesar (Ward Hill, MA, USA) through a gracious gift from Dr. Shawn Campagna's research group (University of Tennessee, Knoxville, TN, USA).

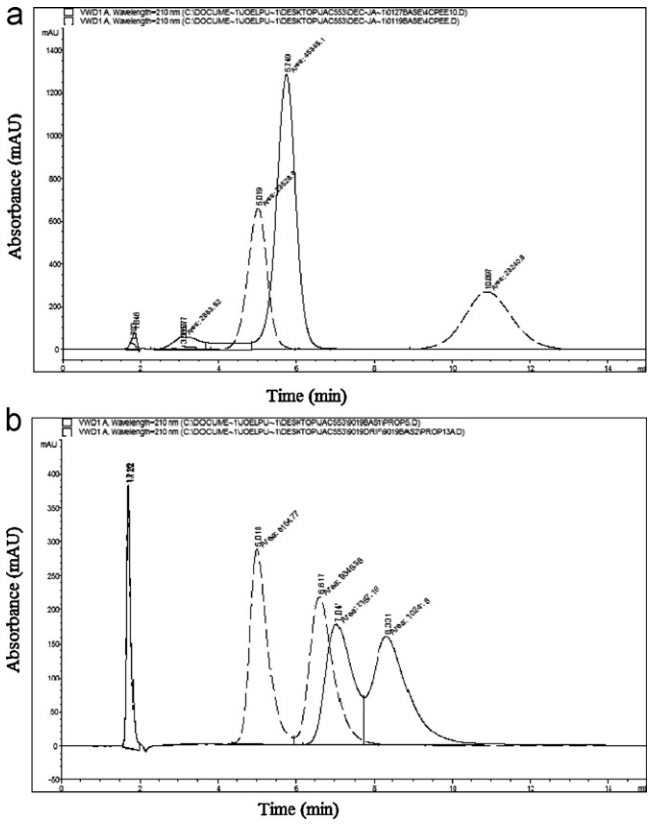


**Fig. 2.** (a) The solid line represents the separation of a 1.0 mg/mL injection of the 4CPEE racemic mixture without ESA treatment to the stationary phase. The broken line represents the separation of a 1.0 mg/mL injection of the 4CPEE racemic mixture after the ESA treatment to the stationary phase. Conditions: 40 °C, 90/10 (v/v) hexanes/ACS reagent grade alcohol, 6 bar, 1.0 mL/min flow rate, 20 μm CHIRALPAK AD stationary phase, and 4.6 × 150 mm column. (b) The solid line represents the separation of a 0.5 mg/mL injection of the propranolol racemic mixture without DIPEA treatment to the stationary phase. The broken line represents the separation of a 0.5 mg/mL injection of the propranolol racemic mixture after the DIPEA treatment to the stationary phase. Conditions: same as Fig. 1a.

### 2.2. Equipment

An Agilent (Santa Clara, CA, USA) HPLC 1090 was used for both the method development of preparative separations and actual preparative chromatographic separations. The HP 1090 instrument used was equipped with three pump heads. The first pump head was set to deliver the stream of Hexanes accounting for 90% of the mobile phase flow rate to the column. The second pump head was set to deliver the stream of ACS reagent grade alcohol to the column (10% of the total mobile phase flow rate). The third pump head was used to deliver the feed solutions to the column when needed. The mixing chamber between the pumps and the column ensures a consistent mix of the mobile phase reaching the column inlet. By adjusting the time during which the third pump was in operation, specific quantities of each racemic mixture could be delivered to the column. In this way the HP 1090 simulated the preparative method commonly used in large scale purification.

### 2.3. Columns

The analytical and preparative columns were received from Chiral Technologies (West Chester, PA, USA). The analytical column (4.6 mm × 150 mm) had been used in previous studies of the memory effect. The preparative column (10.0 mm × 100 mm) was used in previous measurements of isotherms by frontal analysis of the Tröger's base enantiomers by Mihlbachler et al. [13,17]. Both columns contained 20 μm CHIRALPAK AD stationary phase. Neither column was previously exposed to acid or base modifiers except to the documented mobile phases described in previous publications. In particular, the analytical column was exposed to ESA, ethanol, DIPEA, and Hexanes as mobile phases and additives. The analytical column was also exposed to the racemic mixtures of TSO, 4CPEE, 4-chlorophenylalanine methyl ester (4CPME), ketoprofen, propranolol, and Tröger's base. The preparative column was previously exposed to isopropyl alcohol, methanol, and Tröger's base. The guard columns used were packed by Chiral Technologies and contained the same CHIRALPAK AD stationary phase as the columns.

## 3. Procedures

Both the analytical and the preparative columns were treated by injection of the same solution of ethanesulfonic acid. This injection solution was made of ≃10 mg ESA per 100 mL of a 9:1 (v/v) hexanes/ethanol solution. The analytical column was then injected 20 times with 100 μL of the ESA solution. The preparative column was injected 65 times with the same ESA solution. In this way, the two columns were exposed to the same ESA/stationary phase mass ratio. In both columns the injection sequence was: an injection of 4CPEE (concentration of 1.12 mg/mL) followed by the ESA injection. This sequence was repeated every 30 min.

The treatment of the columns with base was similar. The injection solution was made of ≃10 mg DIPEA per 100 mL of a 9:1 (v/v) hexanes/ethanol solution. The analytical column was then injected 7 times with 100 μL of the DIPEA solution. The preparative column was injected 23 times with the same ESA solution. In this way, the two columns were exposed to the same DIPEA/stationary phase mass ratio. In both columns the injection sequence was: an injection of propranolol (concentration of 0.504 mg/mL) followed by the DIPEA injection. This sequence was repeated every 30 min. The number of DIPEA injections was decreased due to the fact that less of the DIPEA was required to create the BME. This could be due to the fact that DIPEA diffuses faster through the polymer than ESA or that fewer sites need to be activated by the base mobile phase modifier.

The preparative injection solutions were made with 9:1 (v/v) solutions of hexanes/ACS reagent grade alcohol in 50 mL volumetric flasks. The Tröger's base was made at a concentration of 10.06 mg/mL. The TSO was created at a concentration of 10.08 mg/mL. Neither of these racemic mixtures were near the maximum concentration due to the limitations of the UV/Vis detector and the feed pump. These lower concentrations were chosen to insure maximum loading without causing the loss of selectivity or resolution. The two remaining racemic mixtures were loaded at maximum mobile phase concentrations of 7.67 mg/mL for 4CPEE and 0.503 mg/mL for propranolol.

The resolution between the two enantiomers was used to characterize the degree of column loading, which ensured that results on the analytical and the preparative columns could be easily compared. The traditional method of characterizing the degree of loading of preparative columns by the sample size that gives a touching band separation would only have allowed for the determination of a maximum loading under the memory effect conditions [18]. The sample sizes providing resolutions of 1.0 and 1.5 (calculated by the Agilent ChemStation software) provide proper estimates of the influence of the equilibrium isotherms of the two enantiomers. This also ensures that the same separation is achieved on both columns.

The loading method for the TSO racemic mixture was optimized at the BME conditions. During the maximum loading of TSO all the active sites which influence the selectivity are used due to the overloading of the adsorption sites. If the BME influenced different active sites than the AME, a difference in the loading of TSO would be noticed. On the other hand, if the active sites for the neutral enantiomers were unaffected by either the AME or the BME, no loading differences would be detected.

The Tröger's base enantiomers lose separation selectivity under conditions of maximum AME [12]. Only after continuous flushing of the column with the unmodified (neutral) mobile phase or treating the column with a base solution does the separation of this racemic mixture return to the origin condition. The separation of the Tröger's base enantiomers does not require a base modifier; yet under analytical conditions the addition of a base modifier produces a higher resolution. As a result, this racemic mixture is influenced by the presence of both the AME and the BME. Measuring the changes in selectivity caused by the memory effect at the preparative conditions provides a useful assessment of their influence under preparative conditions.

A previous study showed that the enantiomers of 4CPEE could be separated under analytical conditions only if an acidic modifier was present in the mobile phase or if the column was under the AME [12]. The requirement of a mobile phase modifier should apply under preparative conditions as well. The systematic measurement of the resolutions of preparative separations of the 4CPEE enantiomers achieved under AME conditions provides an assessment of the influence of AME on the separation of enantiomers requiring acidic conditions. The stability of the AME under preparative conditions could also be derived from changes of the resolution over time.

The enantiomers of propranolol can be separated under analytical conditions only with a base modifier or under the BME [19]. As a compliment to that of 4CPEE, the preparative separation of propranolol assessed the influence of BME on the separations of enantiomers requiring basic conditions. The evaluation of the preparative separation of propranolol measured the stability of BME.

The analytical conditions of the separations are listed in Tables 1 and 2, where Table 1 represents the separation using a resolution of 1.0 with both the BME and AME conditions and Table 2 represents the separation using a resolution of 1.5 with the BME and AME conditions. Due to the large amounts of feed required to overload the column, the feed pump was set to operate at a flow rate 50% larger than the mobile phase flow rate. The total feed amount injected on the analytical column during each separation is also given in Tables 1 and 2. The temperature of the columns was held at 40 °C during all the experiments. Detection was carried out at the best wavelength for each separation (see Tables 1 and 2). The back pressure of the column was initially 8 bar for the analytical column and 6 bar for the preparative column. During the ESA loading, the inlet pressure of the analytical column did continue to increase up to the maximum recommended (70 bar) [20] for this stationary phase. Flushing the column with ethanol/DIPEA removed the excessive pressure, and the subsequent separations of TSO and Tröger's base indicated that the column was not damaged during high pressure operations. The permeability drop could have been due to impurities in the feedstock or the accumulation of layers of racemic material on the polymers. The inlet pressure of the preparative column did not exceed 28 bar, indicating that the pressure phenomenon might be due to the loading method. Additional ESA loading studies were carried out to determine the source of the high pressure. During these studies, 1 cm guard columns were

**Table 1**
Preparative separation conditions using a resolution of 1.0.

| | Analytical conditions | | | | Base memory loading | | | | Acid memory loading | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Racemic mixture[a] | 1 | 2 | 3 | 4 | 1 | 2[c] | 3 | 4 | 1 | 2 | 3 | 4 |
| Inj. mass (mg) | 3.85 | 0.94 | 5.96 | 0.064 | 11.2 | X | 17.1 | 0.21 | 11.2 | 1.65 | 17.1 | 0.21 |
| Separation time (min) | 4.5 | 25 | 4.5 | 6.0 | 4.5 | X | 4.5 | 4.5 | 4.5 | 8.0 | 4.5 | 4.5 |
| Detection wavelength (nm) | 272 | 228 | 307 | 235 | 272 | X | 307 | 235 | 272 | 228 | 307 | 235 |
| Theoretical loading (%) | – | – | – | – | 92.3 | X | 91.0 | 102 | 92.3 | 174 | 91.0 | 102 |
| Stability[b] | 75 | 105 | 71 | 60 | 135 | X | 163 | 90 | 112 | 1500 | 1600 | 1690 |
| Productivity (g enantiomer/kg SP/day) | 795 | 35 | 1230 | 13 | 734 | X | 1120 | 14 | 734 | 60.9 | 1120 | 14 |

[a] Loading sequence: 1 is TSO, 2 is 4CPEE, 3 is Tröger's Base, and 4 is propranolol.
[b] Stability measured in column volumes of solvent percolated through the column.
[c] 4CPEE does not separate under the base memory effect condition.

**Table 2**
Preparative separation conditions using a resolution of 1.5.

| | Analytical conditions | | | | Base memory loading | | | | Acid memory loading | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Racemic mixture[a] | 1 | 2 | 3 | 4 | 1 | 2[c] | 3 | 4 | 1 | 2 | 3 | 4 |
| Inj. mass (mg) | 2.34 | 0.441 | 2.94 | 0.0075 | 6.09 | X | 8.83 | 0.019 | 6.09 | 0.445 | 8.83 | 0.019 |
| Separation time (min) | 4.5 | 25 | 5.0 | 6.0 | 4.5 | X | 4.5 | 4.0 | 4.5 | 8.0 | 4.5 | 4.0 |
| Detection wavelength (nm) | 272 | 228 | 307 | 210 | 272 | X | 307 | 212 | 272 | 228 | 307 | 212 |
| Theoretical loading (%) | – | – | – | – | 82.6 | X | 106 | 121 | 82.6 | 100 | 106 | 124 |
| Stability[b] | 62 | 132 | 35 | 60 | 135 | X | 115 | 90 | 112 | 1500 | 1600 | 1690 |
| Productivity (g enantiomer/kg SP/day) | 483 | 16.4 | 546 | 1.2 | 399 | X | 579 | 1.4 | 399 | 16.4 | 579 | 1.4 |

[a] Loading sequence: 1 is TSO, 2 is 4CPEE, 3 is Tröger's Base, and 4 is propranolol.
[b] Stability measured in column volumes of solvent percolated through the column.
[c] 4CPEE does not separate under the base memory effect condition.

used to check the stability of the stationary phase and whether the inlet frits could have been clogged. They were not and inspection of the guard column packing material did not indicate the trapping of impurities. The tests made with the guard columns indicated that the high pressure was due to solubility issues with 4CPEE and ESA.

The adsorption of the Tröger's base was described as a bi-Langmuirian isotherm by Mihlbachler et al. [13]; during these experiments the 4CPEE and propranolol also exhibit complex Langmuirian isotherms. Such isotherms are due to the formation of either self-assembled mono- or multi-layers on the surface of the stationary phase which could be produces by the individual enantiomers or by the racemic mixture.

The resolution was calculated using the Agilent ChemStation software. The selectivity was derived from the peak retention factors. The productivity was calculated by determining the minimum separation time required between the beginning of the first peak's and the end of the second peak's UV signal. The maximum number of possible injections per day was calculated on the basis of 24 h of operation per day. The amount injected was then multiplied by the maximum number of daily injections and normalized to 1 kg of stationary phase, giving the amount of enantiomer produced per kg of stationary phase per day (g enantiomer/kg stationary phase/day) [21], which is the productivity of a specific separation.

## 4. Results and discussion

### 4.1. General observations

The loading capacities of the CHIRALPAK AD under both the AME and BME conditions are given in Tables 1 and 2 for propranolol, Tröger's base, and TSO. Initially, only the loading of 4CPEE and TSO were performed under the AME. Unlike the analytical separation of the 4CPEE enantiomers, continuous overloading the column with 4CPEE reached a steady-state where the selectivity did not decrease. After the production of 4CPEE reached a steady-state and testing of the separation stability was completed, the separation methods optimized for propranolol and Tröger's base were used. The results of these enantiomeric loading separations will be discussed under the corresponding sections below.

The variance associated with method development on the analytical column to the preparative separation is shown in Tables 1 and 2. The two base racemic mixtures actually separate better on the preparative column than with the equivalent method on the analytical column, while the 4CPEE pair separates almost as well and the TSO separates slightly less than expected. The higher than expected loading of the two basic racemic mixtures might be a secondary effect of the memory effect. The preparative column had been used first to separate the Tröger's base pair, then the propranolol racemic mixture, both under overloaded conditions (as shown in Tables 1 and 2). If this is the case, this would indicate that the BME is nearly permanent and could be created by significant concentrations of the racemic mixture themselves, as well as by the mobile phase modifiers, since the preparative column was not exposed to any modifier prior to this preparative study.

The stability of preparative separations is of great concern in the manufacturing and purification of pharmaceuticals. A given unit must be able to continuously produce the same amount of purified enantiomers for long periods of time if a sufficiently economical production is too be obtained. Tables 1 and 2 show the results of making repetitive injections of each racemic mixture on the preparative column.

### 4.2. trans-Stilbene oxide

The TSO loading experiments on the CHIRALPAK AD yield two results. First, neutral molecules are nearly completely unaffected by either the BME (Fig. 3a) or AME (Fig. 3b) at both extremes. In both conditions, the same amount of TSO racemic mixture produces similar separations. No difference can be observed between the productivities under acid or base mobile phase, at similar resolution. In Fig. 3a and b the solid lines represent the resolution of the TSO separation is 1.52 and in Fig. 3a and b the broken lines represent the resolution of the separation is 0.97. This observation leads to the second result: the active sites for chiral recognition of neutral molecules are completely unaffected by acid or base modifiers, even for the preparative scale. These results indicate that a physical conformational change of the amylose tris(3,5-dimethylphenylcarbamate) polymer is unlikely. If the shape of the polymer structure was changed by exposure to the mobile phase modifiers, the location and number of active sites are very unlike to have remained identical. Any change in the three dimensional structure of the polymer would have considerable effect on the chiral environment created by the stationary phase, leading to either an increase or loss of selectivity at the preparative separation level for these enantiomers.

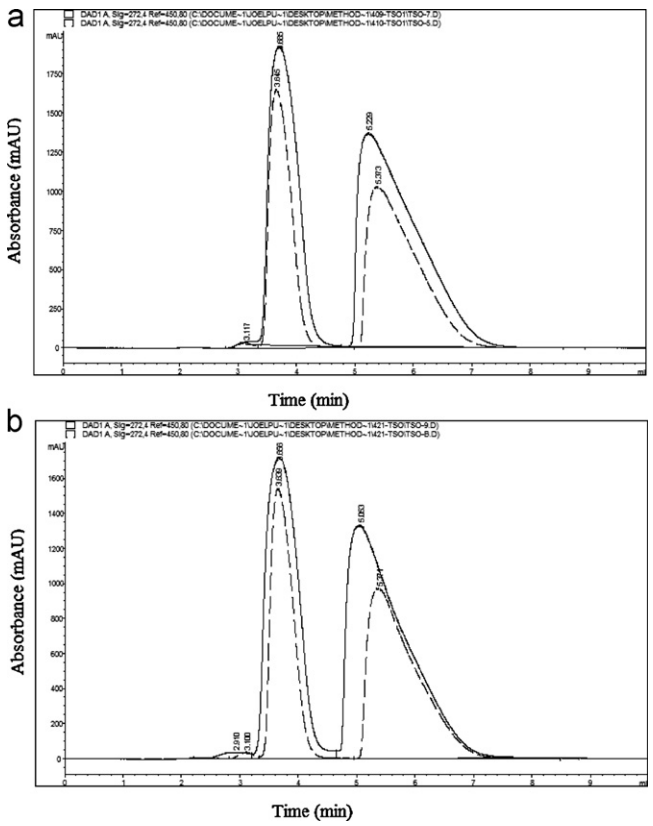


**Fig. 3.** (a) The preparative separation of the TSO racemic mixture after the column was exposed to DIPEA. The solid line represents the separation when the resolution was 0.97, the selectivity was 1.97, which included an injection of 11.2 mg of TSO racemic mixture and a 4.5 run time giving a loading capacity of 734 g racemate/kg CSP/day. The broken line represents the separation when the resolution was 1.52, the selectivity was 2.05, which included an injection of 6.09 mg of TSO racemic mixture and a 4.5 min run time giving a loading capacity of 399 g racemate/kg CSP/day. Conditions: 40 °C, 90/10 (v/v) hexanes/ACS reagent grade alcohol, 6 bar, 2.6 mL/min flow rate, 20 μm CHIRALPAK AD stationary phase, and 10 × 150 mm column. (b) The preparative separation of the TSO racemic mixture after the column was exposed to ESA. The solid line represents the separation when the resolution was 0.97, the selectivity was 1.97, which included an injection of 11.2 mg of TSO racemic mixture and a 4.5 run time giving a loading capacity of 734 g racemate/kg CSP/day. The broken line represents the separation when the resolution was 1.52, the selectivity was 2.05, which included an injection of 6.09 mg of TSO racemic mixture and a 4.5 min run time giving a loading capacity of 399 g racemate/kg CSP/day. Conditions: same as Fig. 2a.

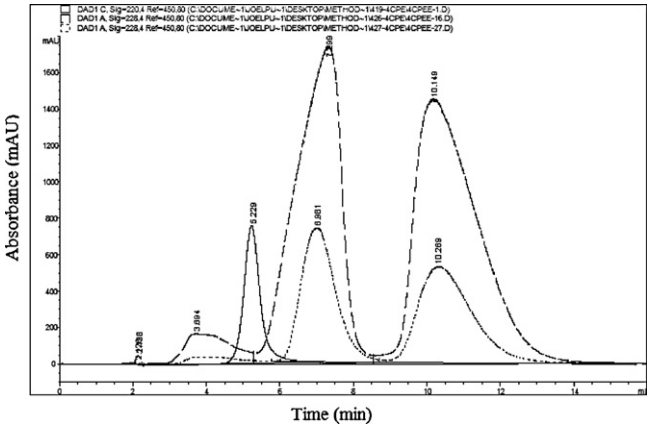


**Fig. 4.** The solid line represents the single peak eluting from a column with no exposure to ESA or DIPEA when an injection of 1.0 mg/mL of the 4CPEE racemic mixture was injected on the CHIRALPAK AD stationary phase. Conditions: same as Fig. 2a. The broken line represents the preparative separation of the 4CPEE when ESA is added to the mobile phase. The resolution was 1.01 and the selectivity was 1.56, with an injection of 1.65 mg and a 9.0 min run time giving a loading capacity of 60.7 g racemate/kg CSP/day. Conditions: 40 °C, 90/10 (v/v) hexanes/ACS reagent grade alcohol with 0.05% ESA, 6 bar, 2.6 mL/min flow rate, 20 μm CHIRALPAK AD stationary phase, and 10 × 150 mm column. The dotted line represents the preparative separation after treatment of the stationary phase with ESA and allowing for the equilibration to occur. The resolution was 1.47 and the selectivity was 1.68, with an injection of 0.45 mg of the 4CPEE racemic mixture and an 8.0 min run time giving a loading capacity of 16.4 g racemate/kg CSP/day. Conditions: Same as Fig. 2a.

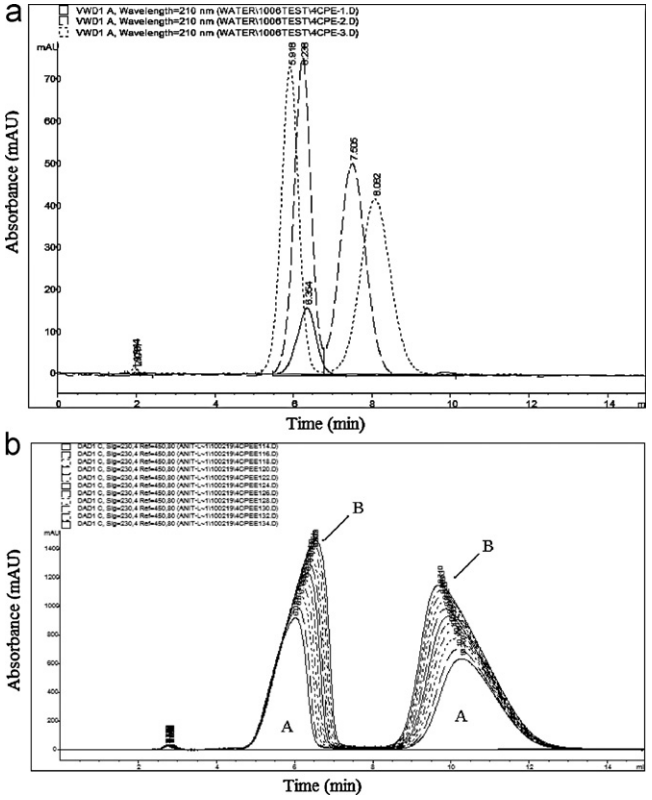


**Fig. 5.** (a)The first injection of the 4CPEE racemic mixture (solid line) on a column which is naïve to this compound will not completely elute from the column, even under analytical conditions. All 3 injections had identical volumes injection of the same concentration, yet 4CPE-1 did not completely elute from the column. Areas counts as measured by Agilent ChemStation software: 4CPE-1 = 5506.3 mAU s; 4CPE-2 = 46,269.1 mAU s; 4CPE-3 = 48,244.5 mAU s; conditions: same as Fig. 2a. (b) The loading profile of the 4CPEE racemic mixture at preparative conditions. The smallest injected mass of racemic mixture was 0.028 mg (labeled A) represents a purely analytical injection of 1 mg/mL at 28 μL ejected on the column. The largest peaks (labeled B) indicate the injection of 150 μL at a concentration of 7.93 mg/mL. The first peak's increased retention as the injection mass increased may indicate that the first eluting enantiomer is forming mono-layers on the surface of the stationary phase.

The stability of this preparative separation was tested by percolating over 125 column volumes through the column, corresponding to 350 mL of mobile phase, which took more than 2 h. The separation was not expected to drift, due to its status as a standard separation. The results under both the AME and the BME merely strengthen this concept; TSO shows no influence of either memory effects. Similar to the analytical conditions, the preparative separation of the TSO racemic mixture under both the AME and BME indicates that the separation of racemic mixtures which do not require mobile phase modifiers can be carried out identically under these conditions.

### 4.3. 4-Chlorophenylalanine ethyl ester

Fig. 4 illustrates the results of the preparative separation of 4CPEE under three different mobile and stationary phase conditions. In Fig. 4 the solid line represents a single peak for both of the 4CPEE enantiomers due to the lack of acidic modification of the either the mobile phase or stationary phase. In Fig. 4 the broken line represents the addition of ESA to the mobile phase and the continuous percolation of this acidic solution through the stationary phase created an acidic environment which allowed for the separation of the racemic mixture. Finally, in Fig. 4 the dotted line represents the chiral environment having been activated by the previous exposure of the column to ESA; yet, the present mobile phase condition was identical to the original solid line chromatogram. In this new activated environment, a steady-state was reached. The steady-state condition (Fig. 4, dotted line) did not provide the same selectivity, resolution, and productivity as the one obtained when the acidic modifier was used (see Fig. 4, broken line), but did have one important advantage. When using modifiers, such as ESA, in preparative separation, the modifier is concentrated with the individual enantiomer at the same time the product is being recovered. Retaining the mobile phase modifier can have undesired effects on the purified enantiomers. The actual mass of modifier collected will affect the final mass of product recovered. For example, in the preparative separation of 4CPEE (using 0.05% ESA in the mobile phase), it was estimated that over 110 ppm of ESA was collected with the first enantiomer per day of operation and over 190 ppm of ESA per day for the second enantiomer. This addition of the mobile phase modifier contaminated the purified product of the separation. Also, this concentration of the mobile phase modifier could be hazardous to the purified enantiomers. Undesired effects of the increased modifier concentration may create a pH level that promotes the degradation of the product, causes epimerization, or increases the likelihood of the formation of ethanesulfonic esters.

The interaction of the 4CPEE racemic mixture on the surface of the chiral stationary phase indicates that, even at the analytical scale, layers of at least one enantiomer may form. In Fig. 5a the first injection of 4CPEE racemic mixture on the column does not fully elute from the column, since the elution peaks of subsequent injections of the same volume and concentration have much larger areas, as measured by Agilent's ChemStation software. The first injection measured 5506.3 mAU s while the second (Fig. 5a, broken line) and third injections (Fig. 5a, dotted line) measured 46,269.1 and 48,244.5 mAU s, respectively. This phenomenon occurred numerous times when the column had been fully flushed with excessive mobile phase. At the preparative scale the formation of enantiomeric layers can been seen in Fig. 5b, which represents the loading profile of the 4CPEE racemic mixture, with the lowest injection mass starting at 0.028 mg (labeled 'A' in Fig. 5b) and increasing up to an injection of 1.19 mg (labeled 'B' in Fig. 5b) of

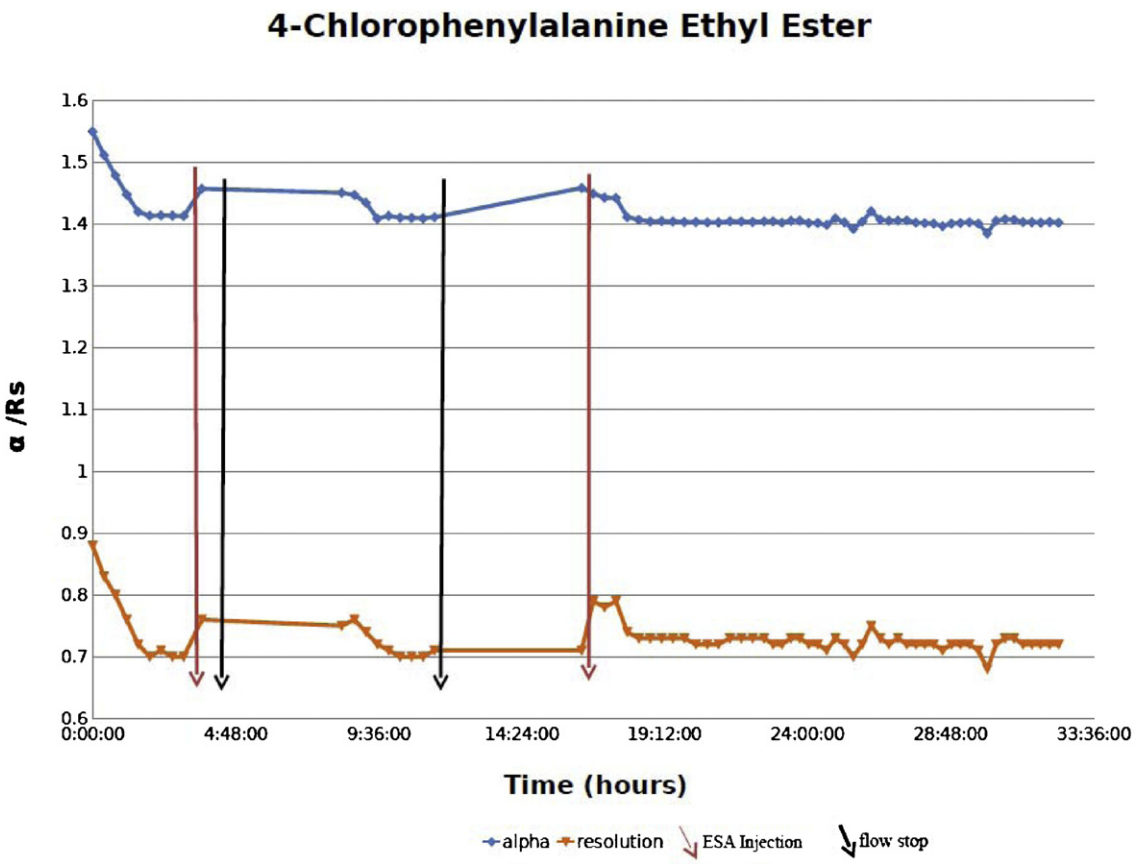


**Fig. 6.** Overloading stability results for the 4CPEE racemic mixture. Each point represents an injection of 100 μL of the mixture. The slight change in selectivity and resolution between the 9th and 19th h maybe due to the slightly change in concentrate for the 4CPEE racemic mixture used. The first 4 h show the decline in selectivity and resolution after removing ESA from the mobile phase. At 3.5 h 100 μL of 10.6 mg/mL ESA was injected onto the column and a single injection of 4CPEE racemate was injected before the flow was stopped. When the flow was started again (2nd red arrow) the selectivity and resolution began to decrease. From the 19th h until end of the experiment (near 33 h) the selectivity and resolution remained constant. This last time period represented nearly 800 column volumes. Conditions: same as Fig. 2a. The black horizontal lines indicate where the mobile phase flow rate was temporarily stopped. The red horizontal arrows indicate where 100 μL injections of ESA were introduced. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

material. The retention of the first eluting enantiomer increases as a larger injection mass was introduced to the column. This type of loading profile is considered to not fit the simplified Langmuir model and indicates that enantiomeric layers are forming on the surface of the stationary phase [22].

The formation of a steady-state 4CPEE separation (see Fig. 6) takes place after only 5 injections. During this experiment the selectivity and resolution of the 4CPEE racemic mixture did not change. The extrapolation of this data indicates that the steady-state remains unchanged even after 3000 column volumes were flushed through the column, corresponding to 50 continuous hours of operation. Further investigation is required to determine the optimum length of time between stationary phase treatments with ESA.

### 4.4. Tröger's base

The separation of the Tröger's base racemic mixture was greatly influenced by the mass injected without regard to the addition of any mobile phase modifiers. For example, when 8.85 mg of the racemic mixture was injected onto the column a resolution similar to Fig. 7a (solid line) was obtained. When the injected mass was increased to 17.5 mg, as seen in Fig. 7a (broken line), the retention times, selectivity, and resolution decreased. The analytical separation was also influenced by the AME and BME, even though the separation was only eliminated at the largest acid mobile phase modifier concentration. As the chiral environment within the polymer structure was influenced by an acidic modifier, the separation of the Tröger's base enantiomers decreased in efficiency and resolution. The retention factors of the enantiomers increased while the peaks broadened when exposed to the increase in the acid mobile phase modifier (see Fig. 7b, solid line). Yet once the ME reached a steady-state – where the preparative separation of the 4CPEE enantiomers remained constant – the loading of the Tröger's base was identical to the separation under the maximum BME (see Fig. 7b, broken line). Two hypotheses remain to be investigated. First, self-assembled layers of enantiomers form, thereby improving the chiral environment, and sustaining the Tröger's base preparative separation. Second, all the active sites required to create the Tröger's base selectivity may have been activated solely by the mobile phase modifiers.

The stability of the Tröger's base preparative separation was tested for 225 column volumes, requiring nearly 4 h. With the Tröger's base, additional volume was used to ensure that the memory effects influence on this loading was accounted for.

### 4.5. Propranolol

The separation of the propranolol racemic mixture is an example of the BME improving a separation upon the addition of a base modifier to the analytical column. Fig. 2b (solid line) shows the separation obtained without a treatment of either an acid or base

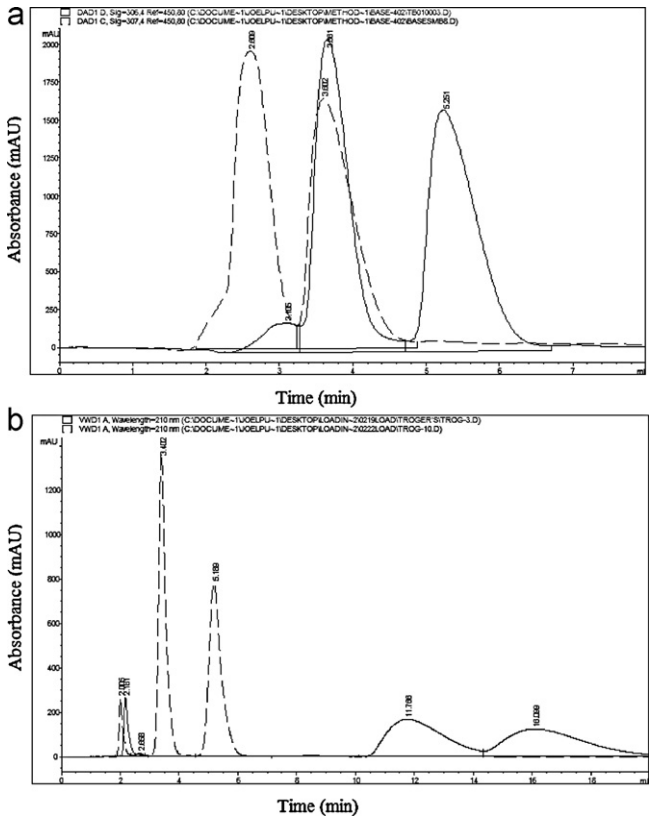


**Fig. 7.** (a) The preparative separation of the Tröger's Base racemic mixture after the column was exposed to DIPEA. The solid line represents the separation when the resolution was 1.53, the selectivity was 2.11, which included an injection of 8.83 mg of Tröger's Base racemic mixture and a 4.5 run time giving a loading capacity of 579 g racemate/kg CSP/day. The broken line represents the separation when the resolution was 1.02, the selectivity was 1.99, which included an injection of 17.1 mg of Tröger's Base racemic mixture and a 4.5 min run time giving a loading capacity of 1,120 g racemate/kg CSP/day. Conditions: same as Fig. 2a. (b) The solid line represents the separation of the Tröger's Base racemic mixture near the maximum ESA loading condition. The broken line represents the separation of the Tröger's Base racemic mixture near the maximum DIPEA loading conditions. Conditions: same as Fig. 2a.

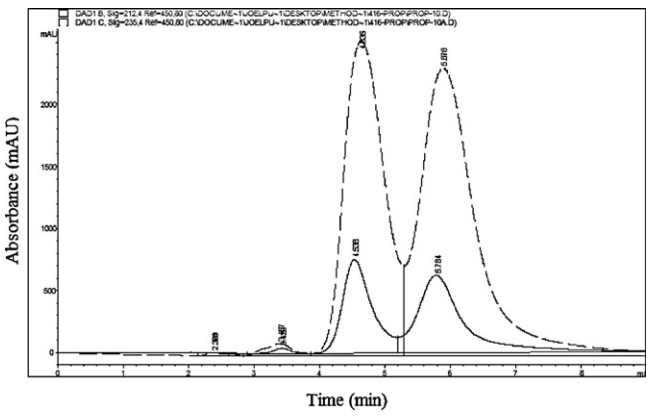


**Fig. 8.** The preparative separation of the propranolol racemic mixture after the column was exposed to DIPEA. The solid line represents the separation when the resolution was 1.37, the selectivity was 1.47, which included an injection of 19.6 μg of the propranolol racemic mixture and a 4.0 run time giving a loading capacity of 1.45 g racemate/kg CSP/day. The broken line represents the separation when the resolution was 0.91, the selectivity was 1.47, which included an injection of 206 μg of the propranolol racemic mixture and a 4.5 min run time giving a loading capacity of 13.5 g racemate/kg CSP/day. Conditions: same as Fig. 2a.

modifier to the mobile or stationary phase. When the column was treated with an acid modifier no separation of the propranolol racemic mixture occurred. Unlike the AME, which slowly reached a steady-state, the propranolol preparative separation indicated that a steady-state may have been reached instantaneously. Once the column had been exposed to the base modifier, the separation continuously performed slightly better. It should be noted that in the preparative separation, this racemic mixture also showed distinct evidence of a complex Langmuirian isotherm. For example, the increase in loading from 19.6 mg, with a resolution of 1.37, to the loading of 206 mg, with a resolution of 1.0, did not change the selectivity of the separation (see Fig. 8, solid line and broken line). This data, combined with results from the Tröger's base and 4CPEE of complex Langmuirian isotherms, may indicate that the formation of enantiomeric layers on the surface of the polymer is required prior to the influence of the memory effect being observed.

The results of the stability test of the preparative separations indicate that propranolol can be separated after the column has been exposed to an acid modifier. Combining the observation that Tröger's base also separates under an AME steady-state suggests that the BME could be permanent. The total column volume between the addition of the base modifier and the final separation of propranolol exceeded 3375 column volumes, equivalent to 56 h of continuous operation.

## 5. Conclusions

Three issues arise when separations developed using amylose tris(3,5-dimethylphenylcarbamate) as the stationary phase are transferred from analytical to broader columns. The first problem is related to the scaling-up of methods. Without the memory effect, scaling separations from analytical to larger columns requires only calculations to adjust for the flow rates, column diameter, and amount of stationary phase used in the larger column. The memory effect phenomenon adds an additional element of complexity and an additional factor that must be corrected for during the scale-up process. A second issue arising when there is a memory effect is a concern or separation stability. In previous studies of the memory effect, it was found that analytical columns slowly lose the capacity to separate specific racemic mixtures that require either the BME or AME condition [12]. Finally, the use of mobile phase additives can be detrimental to the recovery of preparatively purified enantiomeric materials. In this study these issues have been addressed.

The scaling of chromatographic methods from analytical to preparative columns packed with an amylose tris(3,5-dimethylphenylcarbamate) stationary phase was difficult to carry out in the past due to the possilbe presence of the memory effect. In this study, a method was used to determine the correction factor. Using the 4CPEE and propranolol racemic mixture, it was shown that after 1–5 preparative scale injections, a steady-state is reached that is transferable to larger columns with excellent results. However, the reproducibility of any method should always be determined at the analytical level prior to the actual transfer to the preparative column due to the additional amount of product, solvent, and time needed to create the steady-state on larger columns.

The practice of using either base or acid mobile phase additives sometimes improves the preparative separation used to purify enantiomers. Removing these additives from the concentrated product, however, can lead to numerous additional concerns. The additives are not as easy to evaporate as the mobile phase, may cause the degradation of the products or the formation of sulfonic acid impurities. By using the memory effect of these mobile phase additives, instead of the additives instead, these undesirable conditions can be minimized.

The steady-state condition reached after a small number of preparative scale injections is more stable than the analytical sepa-

ration data would suggest. This can be understood if it is recognized that a certain amount of the racemic mixture is adsorbed to the active sites of the stationary phase. Thus, overloading the column with preparative injections requires a continuous resupply of the stationary phase surface with the appropriate active material ensuring that the proper chiral environment remains available to maintain the desired separation. In this study, the preparative separation of the 4CPEE racemic mixture was carried out for more than 14 continuous hours without detecting changes in the selectivity or resolution. In practice the preparative separation would need to be tested to determine the optimum length of time that the separation could be continued before additional treatments of the stationary phase with modifiers would be recommended. Under analytical conditions, the amount injected does not resupply the chiral environment with the proper active material, hence the loss of separation over an extended period of time.

Additionally, this study showed that the resolution of neutral racemic mixtures are unaffected by the presence of either the AME or BME. Preparative separations of Tröger's base and TSO retained the same resolution and selectivity at both the BME and AME steady-states. This result suggests that all the active sites required for the separation of these racemic mixtures remain regardless of either memory effect. As a result, a neutral CHIRALPAK AD column is unnecessary since the neutral enantiomeric pairs will perform as well under any column condition.

The final observation made is the stability of the BME. Racemic mixtures requiring a base modifier in the mobile phase can be separated on the CHIRALPAK AD column even after the modifier has been removed. Under the maximum degree of AME influence, these racemic mixtures requiring a base modifier are not resolved. Yet after a steady-state condition which is capable of separating acid requiring racemic mixtures has been achieved, these racemic mixtures requiring a base modifier can be separated with the same selectivity and resolution as under BME conditions. This suggests that the active base sites are only eclipsed by the presence of an acid modifier and that when the acid modifier is no longer present, the activated base sites are available again.

Once understood, the memory effects can be put to good use. The number of racemic mixtures that can be preparatively separated on CHIRALPAK AD becomes larger, and the need to store additional expensive columns in a laboratory can be eliminated or reduced.

## Acknowledgments

This work was supported in part by the cooperative agreement between the University of Tennessee and the Oak Ridge National Laboratory. We would like to thank Chiral Technologies (West Chester, PA, USA) for providing the stationary phase used in these studies. We thank Chiral Technologies for packing the columns used.

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