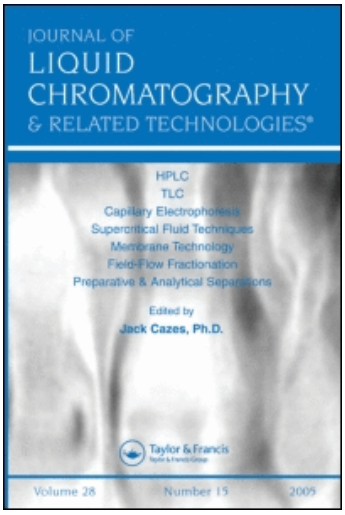

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ABSTRACT

A method for the enantiomeric separation and direct detection of *trans*-2-aminocyclohexanol by high performance liquid chromatography (HPLC) with evaporative light scattering detection (ELSD) using a crown ether column has been developed. The influence of mobile phase composition on the separation was investigated in detail. It was found that enantiomeric separation could be achieved when a strong chaotropic counterion, such as TFA, is used as the mobile phase modifier. Organic modifiers, such as methanol, influence the retention times, but have no effect on the separation factor. Column temperature plays an important role on the

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separation. The Van't Hoff plot reveals that the interaction mechanism is enthalpy driven. The ELSD sensitivity was also studied as a function of gas pressure, drift tube temperature, and nebulizing gas type. The optimum operation condition was to use air as the nebulizing gas (1.5 psi) with the drift tube temperature ranging from 110–115°C.

Key Words: Enantiomeric separation; *Trans*-2-aminocyclohexanol; Crown ether; ELSD.

INTRODUCTION

In the last two decades, organic synthesis of enantiomerically pure compounds has increased dramatically in the pharmaceutical industry. This increase can be attributed to the heightened awareness that pharmacological and toxicological differences can exist between enantiomers and as a result, chiral separation and analysis have become increasingly important. Chiral separations are still challenging, despite the large number of chiral technological achievements.[1–6] Currently, there are two major methods for achieving chiral separation.[7] One method is indirect and is accomplished by derivatization with an enantiomerically pure agent to form a diastereomer, followed by separation on an achiral stationary phase. The other method is achieved by direct chiral separation of the enantiomers on a chiral stationary phase or through chiral additives in the mobile phase. Separations performed on chiral stationary phase are preferable over separation using chiral additive because of robustness. Separations performed with chiral stationary phases can be accomplished through a number of different mechanisms. One of the mechanisms is inclusion in which the guest molecule is included into the cavity of a host molecule.[7] The exterior of the host molecule generally possesses functional groups that act as steric barriers for the chiral selective interaction. The interaction within the cavity of the host molecule may be hydrophobic (e.g. cyclodextrins[8]) or hydrophilic (e.g. crown ethers[9,10]).

The analyte of interest, 2-aminocyclohexanol, has two chiral centers, thus, there are two pairs of enantiomers: *cis*-2-aminocyclohexanol and *trans*-2-aminocyclohexanol. *Trans*-2-aminocyclohexanol hydrochloride (TACH) [Fig. 1(A)] is an important precursor for a new class of sigma-receptors and anticonvulsant drugs.[9] Enantiomerically pure TACH was often chosen as the starting material vs. the racemic mixture, since chiral purification and analysis of the final product may be costly and time consuming. As a result, the chiral separation of *trans*-2-aminocyclohexanol is critical to the process. Because TACH possesses no chromophore, the analysis of this compound usually involves derivatization with a UV absorption moiety (e.g. 2,4-dinitrobenzoyl

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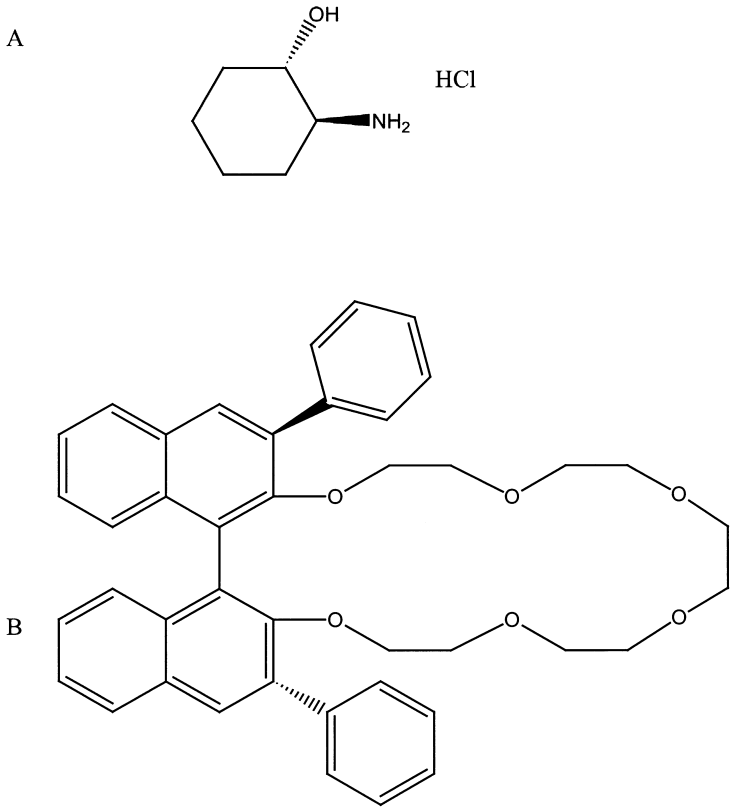

Figure 1. Chemical structures of *trans*-2-aminocyclohexanol hydrochloride (A) and crown ether stationary phase (B).

chloride) followed by separation on a chiral column. The derivatization reaction incorporates a rigid phenyl moiety into the structure, thus, not only enhances the UV absorbance, but also facilitates the chiral recognition due to the steric hindrance of the phenyl group.[10] However, a direct separation and detection method should be more straightforward and robust. Since TACH has primary amine functionality, a crown ether stationary phase can be utilized.[11]

It is known that crown ethers can bind alkylamines, as well as inorganic cations, such as potassium or sodium.[11] The leading interaction between the crown ether and the primary amine is based on hydrogen bonding between the hydrogen atoms of the amino group and the crown ether's oxygen lone pair electrons. Cram and co-workers took advantage of this unique property of

crown ethers to perform enantioseparations on a number of alkylammonium compounds.[12–17] It was discovered that bulky groups, such as binaphthyl groups grafted onto the crown ether, could provide steric barriers and induce enantioselective interactions with the guest molecule.[12] The use of crown ethers for enantioseparations with liquid chromatography was introduced later by Cram and co-workers.[18–20] Shinobo and co-workers found that chiral separations could be achieved on a reversed phase column that was dynamically coated with a chiral crown ether.[22] The structure of the chiral crown ether they used is shown in Fig. 1(B). This crown ether column is commercially available (Crownpak CR) and has been used to separate amines, amino alcohols, amino acids, and amino esters.[23–26]

The lack of a chromophoric functional group within TACH makes the use of evaporative light scattering detection (ELSD) necessary for direct analysis. Evaporate light scattering detection is derived from early work by Charlesworth and McRae.[27] Unlike UV and fluorescence detection, ELSD is a universal detector suitable for analytes with no chromophores.[28–33] This detector is a transport detector and not a material conveyor, it uses a scavenger gas stream to carry the effluent to the detector.[34] The effluent is nebulized immediately at the stream of warm gas. The solvent vaporizes and leaves a cloud of particles, as well as the non-volatile residues in the solvent. The analyte must have a higher vapor pressure than the mobile phase to ensure that it is left in the clouds of particles and not vaporized. These particles are transported along the gas stream across an internal light beam. The amount of scattered light collected on a photomultiplier is a measure of the amount of non-volatile solute in the effluent stream. During the vaporization of the solvent, the size of droplets keeps shrinking until a final volume is reached. The final volume is proportional to the analyte concentration. These principles make ELSD a good detection method for analytes with no UV chromophore.

The direct enantiomeric separation of TACH on a Crownpak CR column with ELSD detection is reported in this work. The effect of mobile phase composition and column temperature on the chiral selectivity and the factors that influence the sensitivity of ELSD, such as nebulizing gas pressure, drift tube temperature, mobile phase flow rate, etc., are discussed in detail.

EXPERIMENTAL

Reagents

All reagents used here were of analytical grade. Trifluoroacetic acid (TFA), TACH, acetic acid, and formic acid were obtained from Sigma-Aldrich

(St. Louis, MO). Methanol (MeOH) was purchased from EM science (Gibbstown, NJ). (1S,2S)-*Trans*-2-aminocyclohexanol tartrate was synthesized at Merck & Co., Inc. (Rahway, NJ). Zero air and nitrogen (99.9%+ pure) were from Airgas East (Piscataway, NJ).

Instrumentation

The high performance liquid chromatographic (HPLC) system consisted of an HPLC 1100 pump with degasser, auto sampler, and UV detector from Agilent Technologies (Wilmington, DE). This system also has a column temperature controller. The Crownpak CR column was purchased from Chiral Technologies (Exton, PA). Data acquisition was performed with a Perkin Elmer (Norwalk, CT) Turbochrom Client/Server system. Sample solutions were prepared by dissolving in water. The HPLC separations were performed at 5°C. The mobile phase consisted of 2% TFA in water/methanol 95/5 and the flow rate was 0.3 mL/min, unless otherwise stated.

Light scattering detection was achieved on an Alltech 500 evaporative light scattering detector (Deerfield, IL). Gas pressure and the drift tube temperature of the detector can be fine-tuned. The attenuation was set at 5. Air and nitrogen were both tested as nebulizing gas. Air is the nebulizing gas of choice unless otherwise stated.

The HPLC pump was connected to the UV detector first, and the effluent coming out of the UV detector was introduced into an ELSD detector directly without any splitter in between. The connection tubing between the UV detector and ELSD was kept as short as possible to minimize void volume.

RESULTS AND DISCUSSION

Optimization for the Enantiomeric Separation

Effect of Counterion Chaotropicity and Concentration

To separate primary amines on a crown ether column, the amine site has to be protonated in order to form a hydrogen bond with the oxygen atoms on the crown ether. Thus, only acidic conditions can be used for the separation. Trifluoroacetic acid (TFA), formic acid, and acetic acid were studied as mobile phase additives. It was found that when using formic

acid or acetic acid in water, the enantiomers were not retained on the stationary phase. Varying the concentration of formic acid or acetic acid did not increase retention, nor improve separation. Similarly, no separation could be achieved when using formic acid or acetic acid in 95/5 (v/v) water/methanol or water/acetonitrile. But, when using a relatively high concentration of TFA in water, i.e., 1% or higher, the enantiomers are baseline separated. A typical chromatogram of the separation is shown in Fig. 2.

This observation can be explained by the effect of the counterion's chaotropicity. Chaotropicity is a measure of the ion's ability to influence hydration. Ions that are strongly chaotropic usually have large ionic volume (low charge density) and are easily polarized.[35] An anion with a high chaotropicity is also characterized by high polarizability with a consequent low degree of hydration. Such ions, when ion paring with the analyte molecule, reduce the degree of hydration of the analyte, and thus, facilitate the approach of the analyte to the stationary phase.[36] When separating TACH on a crown ether column under acidic conditions, the amine group of TACH is protonated and forms hydrogen bonds with the oxygen atoms of the crown ether. In addition to the inclusion phenomena, there is also a nonspecific interaction. This interaction occurs between the hydrophobic portion of the TACH and the crown ether. The nonspecific interaction is affected by the chaotropicity of the counteranion, the stronger chaotropic effect, the stronger the interaction. Trifluoroacetic acid (TFA) is a strong acid with a high chaotropic effect,[34–35] the hydrophobic interactions between the analyte and the stationary phase are much stronger in TFA than in acetic acid or formic acid. Enatioselectivity is provided by the interaction of the TACH with the bulky aromatic groups attached to the crown ether. This enantioselective interaction is also enhanced in TFA as the TACH is brought closer to the crown ether by the chaotropic counterion effect. As a result, both capacity factor and separation factor increase with the use of TFA vs. the use of formic acid and acetic acid since TFA is known to be a stronger chaotropic counteranion.[35] Previous studies with cellulose phases[36] or crown ether phases[37] have shown similar effects of the counterion's chaotropicity on chiral separation.

It was also found that the capacity factor and the selectivity factor increase with increasing concentration of TFA (Table 1). This can also be explained by the chaotropic effect of the mobile phase counterion. An increase in TFA concentration will enhance the chaotropic effect; thus, the specific interactions and non-specific interactions were enhanced as well, resulting in the increase of capacity factor and separation factor.

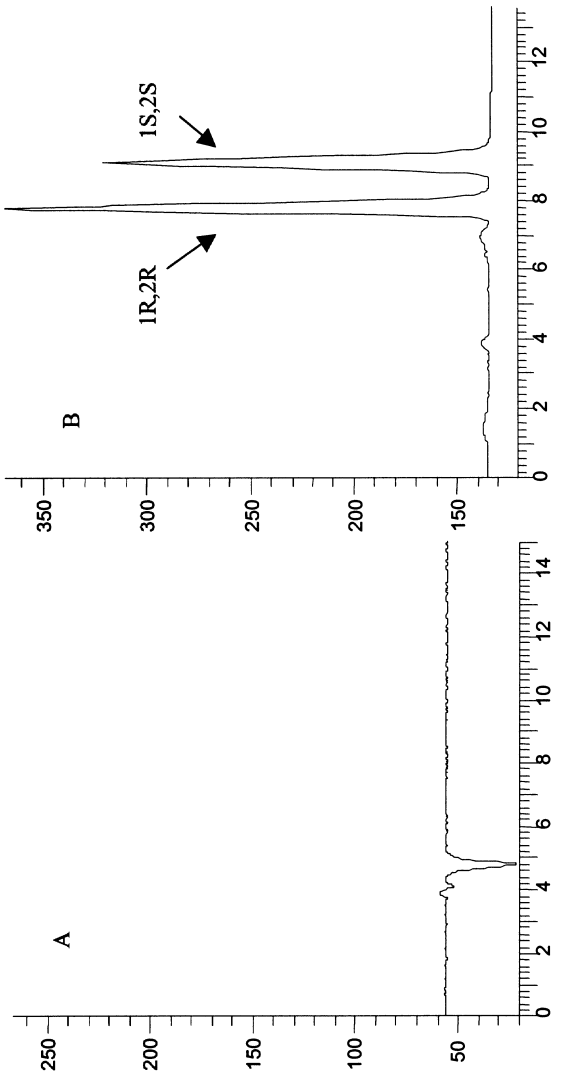

Figure 2. Chiral separation of TACH: Crownpak CR(−) column 4.0 × 150 mm; mobile phase: 2% TFA in 95/5 water/methanol; flow rate: 0.3 mL/min; sample concentration: 0.2 mg/mL, 20 µL injection; detection: (A) UV detection @ 200 nm; (B) ELSD nebulizer temperature 115°C, gas type: air @ 1.54 psi.

Table 1. Effect of TFA concentration on separation factor.

Volume % TFA[a]	$k'1$	$k'2$	Separation factor
0.2	0.56	0.67	1.19
0.4	0.66	0.80	1.21
0.6	0.73	0.89	1.22
0.8	0.77	0.96	1.25
1.0	0.83	1.06	1.28
1.2	0.86	1.11	1.28
1.4	0.97	1.24	1.28
1.6	0.98	1.27	1.30
1.8	1.00	1.32	1.32
2.0	1.02	1.36	1.33
3.0	1.09	1.51	1.39
4.0	1.25	1.80	1.43
5.0	1.41	2.04	1.44

[a]Separation conditions: Crownpak CR(−) column 4.0 × 150 mm; mobile phase: varying amount of TFA in 95/5 water/methanol; flow rate: 0.3 mL/min; sample concentration: 0.2 mg/mL, 20 µL injection; detection: ELSD nebulizer temperature 115°C, air @ 1.54 psi.

Effect of Organic Modifier on the Separation Parameters

Previous research on the effect of organic modifier has demonstrated that $\ln k'$ vs. volume fraction of modifier can be expressed in the following equation:[39]

$$\ln k' = \ln k'_w - S\phi \tag{1}$$

k' is the solute capacity factor at the specific composition of the mobile phase ϕ, and k'_w is the extrapolated k' for pure aqueous mobile phase when $\phi = 0$, and S is a constant. Equation (1) predicts that a plot of $\ln k'$ vs. ϕ will be a straight line with the slope of S and an intercept of $\ln k'_w$. The organic modifier used in this study is methanol and its volume concentration did not go higher than 15%, due to manufacturing constraints on the column. The methanol

concentration varied from 0.0 to 0.14 volume fraction. A plot of $\ln k'$ vs. volume fraction of methanol is plotted in Fig. 3 for both enantiomers. It demonstrated that the retention of both enantiomers decrease with an increase in methanol concentration. The graph also reveals that a plot of $\ln k'$ vs. volume fraction of methanol is not linear. However, two straight lines with different slopes can be extracted from each curve. This indicated the presence of two interactions contributing to the retention of the analyte according to Eq. (1). Increase of the methanol concentration decreases both interactions. The selectivity factor of the enantioseparation was calculated and plotted vs. volume fraction of methanol in Fig. 4. It indicates, that while retention is governed by organic modifier concentration, enantioselectivity is independent of the organic modifier concentration.

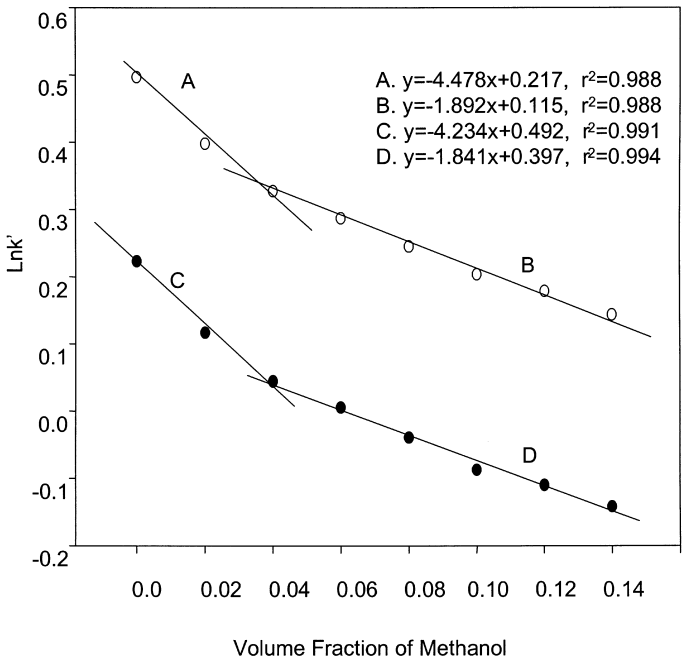

Figure 3. $\ln k'$ vs. volume fraction of methanol in mobile phase. Crownpak CR(−) column 4.0×150 mm; mobile phase: 2% TFA in water/methanol; flow rate: 0.3 mL/min; sample concentration: 0.2 mg/mL, 20 μL injection; detection: ELSD nebulizer temperature 115°C, gas type: air @ 1.54 psi.

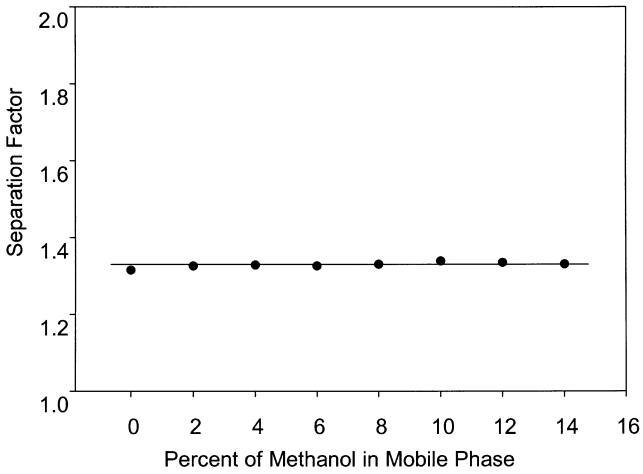

Figure 4. Effect of methanol on selectivity factor. Crownpak CR(−) column 4.0 × 150 mm; mobile phase: 2% TFA in 95/5 water/methanol; flow rate: 0.3 mL/min; sample concentration: 0.2 mg/mL, 20 µL injection; detection: ELSD nebulizer temperature 115°C, gas type: air @ 1.54 psi.

Effect of Column Temperature on the Separation

The capacity factor of a solute is related to the change in partial molar free energy incurred during the transfer of solute between the mobile phase and the stationary phase as shown in the following equation:[40,41]

$$\ln k' = -\left(\frac{\Delta G^0}{RT}\right) + \ln \Phi \tag{2}$$

where Φ represents the phase ratio. The free energy can be broken down into enthalpy and entropic terms to give the van't Hoff equation:

$$\ln k' = -\left(\frac{\Delta H^0}{RT}\right) + \frac{\Delta S^0}{R} + \ln \Phi \tag{3}$$

Consequently, a plot of $\ln k'$ vs. $1/T$ should be linear, with a slope of $(-(\Delta H^0/R)$ and an interception of $(\Delta S^0/R + \ln \Phi)$. Additionally, for a pair of enantiomers,

$$\ln \alpha = -\frac{\Delta\Delta G^0}{RT} = -\frac{\Delta\Delta H}{RT} + \frac{\Delta\Delta S^0}{R} \tag{4}$$

From Eq. (3), one can calculate the difference in free energy and from plots of $\ln \alpha$ vs. $(1/T)$, the difference in enthalpy and entropy between the two enantiomers can be calculated.

In this experiment, the capacity factors of the two enantiomers were measured from 5 to 50°C at 5° intervals using a Crownpak CR(−) column with 2% TFA in water/methanol (95/5) as the mobile phase. Capacity factors for the two enantiomers were measured at different column temperatures, and separation factors were calculated from the capacity factors. The Van't Hoff plots were generated for the two TACH enantiomers and are shown in Fig. 5. It was found that $\ln k'$ vs. $1/T$ was linear, which indicates no change in the retention mechanism when using TFA in water/methanol as mobile phase at 5 to 50°C temperature. The observed $\ln \alpha$ vs. $1/T$ was also plotted (Fig. 6) and the $\Delta\Delta H$ was calculated to be −595.09 cal/mol, and $\Delta\Delta S - 1.66$ cal/(mol K). These results indicate that the chiral selective interaction is predominately enthalpy driven.

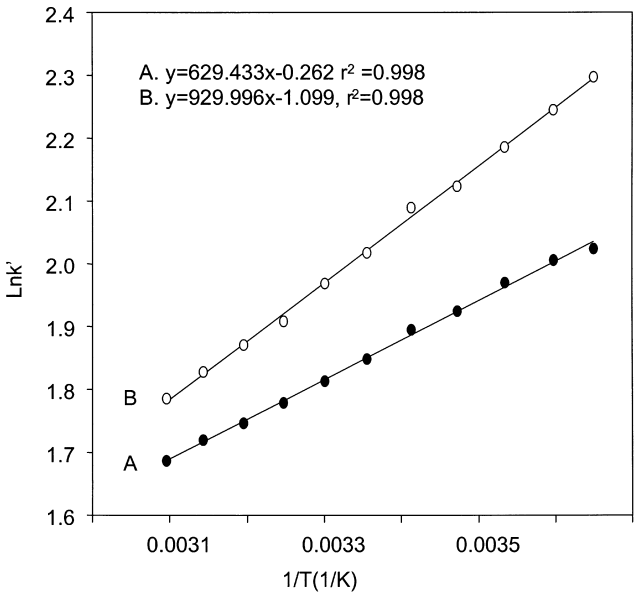

Figure 5. Van't Hoff plots for the chiral separation of TACH. Crownpak CR(−) column 4.0 × 150 mm; mobile phase: 2% TFA in 95/5 water/methanol; flow rate: 0.3 mL/min; sample concentration: 0.2 mg/mL, 20 μL injection; detection: ELSD nebulizer temperature 115°C, gas type: air @ 1.54 psi, ● data for the 1R,2R-enantiomer, ○ data for the 1S,2S-enantiomer.

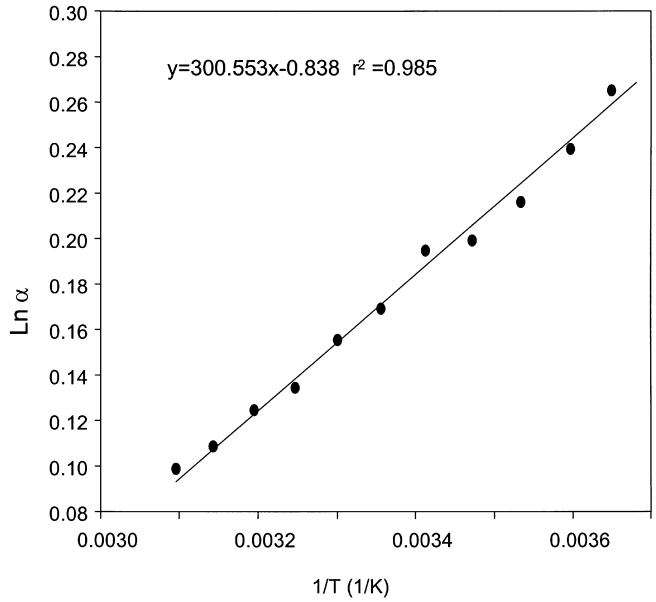

Figure 6. $\ln \alpha$ vs. $1/T$ for the separation of TACH: Crownpak CR(−) column 4.0 × 150 mm; mobile phase: 2% TFA in 95/5 water/methanol; flow rate: 0.3 mL/min; sample concentration: 0.2 mg/mL, 20 µL injection; detection: ELSD nebulizer temperature 115°C, gas type: air @ 1.54 psi.

Optimization of the Evaporative Light Scattering Detection Parameters

Effect of Drift Tube Temperature

Complete evaporation of solvent is very critical for the accurate detection of analyte using ELSD. The kinetics of the heat transfer in the tube between the gas and the nebulized analyte droplets must be fast enough to ensure complete vaporization of solvents.[34,42–43] Thus, the temperature of the drift tube is an important experimental parameter to ensure the total vaporization of the mobile phase, while keeping the analyte in a particle form. If the temperature is too high, error of analysis could occur due to some analyte vaporization with a total loss of signal if complete vaporization of analyte occurs. If the temperature is too low, baseline noise will be very high due to the interference of un-evaporated solvent droplets.[34] In this study, the mobile phase contained high percentile aqueous (95% to 100%). Thus, the drift

tube temperature must be set at levels close to 100°C or higher to ensure complete vaporization of the mobile phase. Different drift tube temperatures ranging from 95 to 130°C were tested. The effect of drift tube temperature to response and baseline noise is demonstrated in Fig. 7. It was found that at 105°C, the analyte gives the highest response [Fig. 7(A)]. The lowest baseline noise level was observed with drift tube temperature at 115 to 120°C [Fig. 7(B)] and the highest signal/noise (S/N) ratio was obtained around 110–115°C [Fig. 7(C)].

Effect of Gas Type and Gas Pressure

To investigate the effect of gas type on the detection, two types of gases were used in this study: air and nitrogen. Gas pressures ranging from 1.0 to 3.7 psi were also investigated, while keeping constant mobile phase flow at 0.3 mL/min and drift tube temperature at 115°C. The effect of gas pressure on the response factor and noise level for both gas types are shown in Fig. 8. It was observed, that the response factor increases and the noise level decreases with decreasing nebulizing gas pressure [Fig. 8(A),(B)]. The average particle size of the solute cloud decreases with increasing gas pressure and the response factor of the analyte decreases as smaller particles give less scattered light.[27] However, if the gas pressure is too low, there could be a problem of the mobile phase being partially vaporized. Under such conditions, the noise level could be high and spikes may start to appear. Thus, S/N does not always favor lower gas pressure. Figure 8(C) demonstrates that there is an optimum gas pressure ($\sim$1.5 psi), which yields the highest S/N. Overall, these studies revealed that the response factor and noise levels were generally higher when air is used as the nebulizer carrier gas.

Effect of the High Performance Liquid Chromatography Mobile Phase Flow Rate on the Response Factor

The effect of mobile phase flow rate on the response factor was also studied, and the results shown in Fig. 9. It was found, that the flow rate should not exceed 0.5 mL/min, due to an exponential increase of noise level with flow rate, making the conditions impractical for any useful application. This phenomenon is relatively easy to understand, since the mobile phase used here contains a very high percentage of aqueous solution with a high vapor pressure. Thus, at high flow rate, the heat transfer in the drift tube is not fast enough to evaporate all the water in the mobile phase, even when the

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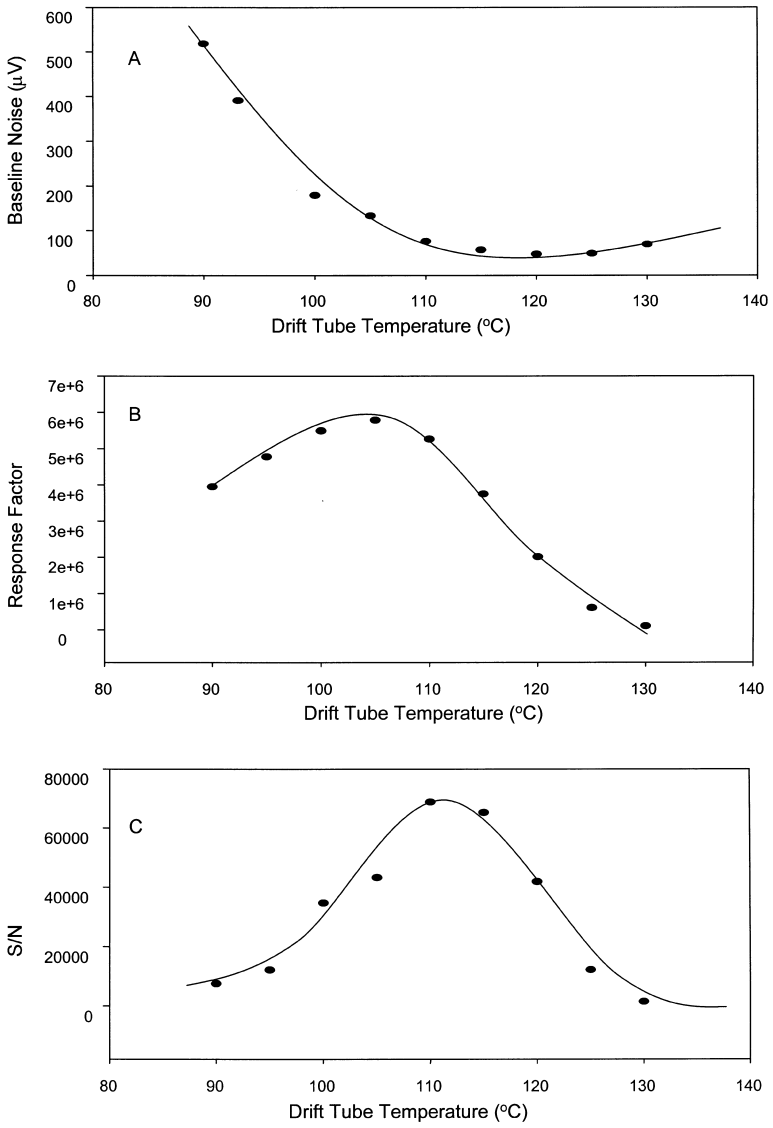

Figure 7. Effect of ELSD drift tube temperature to the baseline noise level (A) and response factor (B). Crownpak CR(−) column 4.0 × 150 mm; mobile phase: 2% TFA in 95/5 water/methanol; flow rate: 0.3 mL/min; sample concentration: 0.2 mg/mL, 20 μL injection; detection: ELSD gas type: air @ 1.54 psi with varying nebulizer temperature.

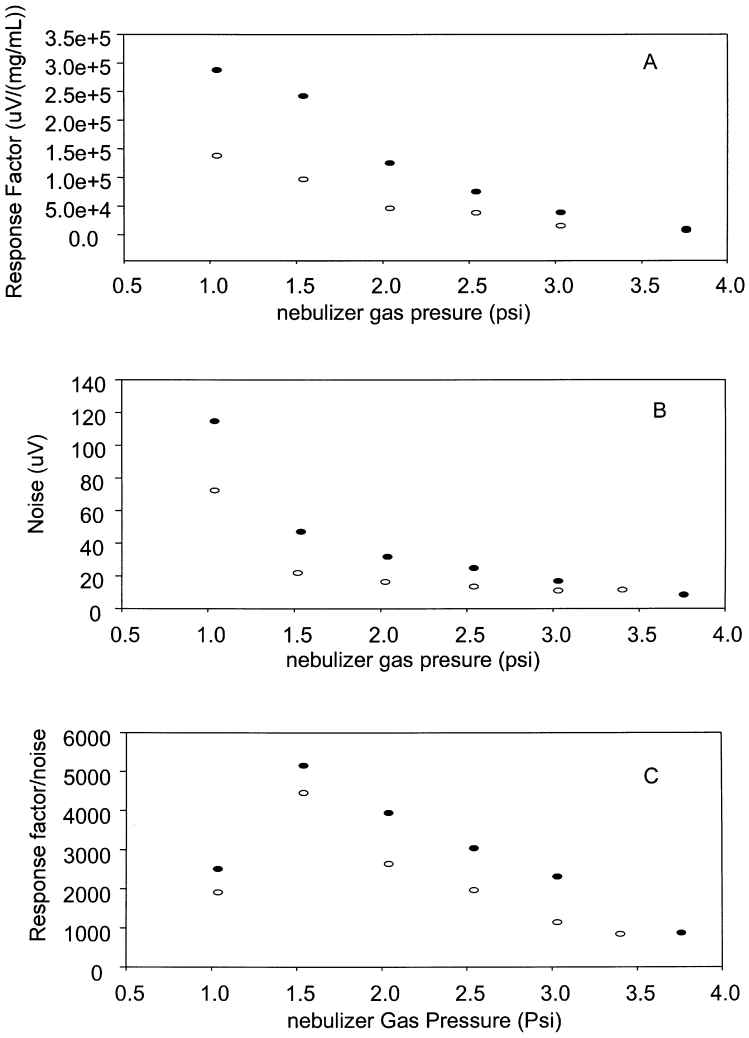

Figure 8. Effect of ELSD gas pressure and gas type to the baseline noise level (A) and response factor (B). Crownpak CR(−) column 4.0 × 150 mm; mobile phase: 2% TFA in 95/5 water/methanol; flow rate: 0.3 mL/min; sample concentration: 0.2 mg/mL, 20 μL injection; ELSD detection: drift tube temperature at 115°C; gas type: ● air, ○ nitrogen.

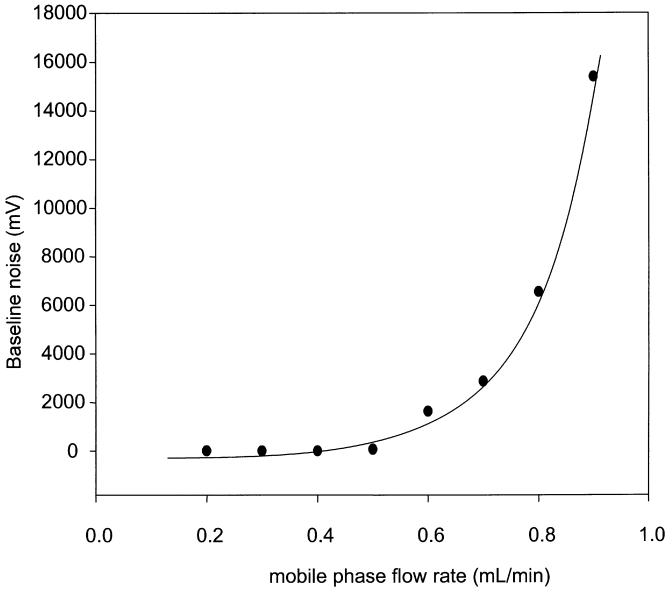

Figure 9. Effect of mobile phase flow rate to ELSD baseline noise. Crownpak CR(−) column 4.0 × 150 mm; mobile phase: 2% TFA in 95/5 water/methanol; sample concentration: 0.2 mg/mL, 20 μL injection; ELSD detection: drift tube temperature at 115°C.

temperature is set at 115°C. As a result, the background noise is extremely high, and the amount of spikes in the background increases tremendously.

CONCLUSIONS

A racemic mixture of *trans*-2-aminocyclohexanol was separated on crown ether coated stationary phase using ELSD detection. It was found, that the capacity and separation factors were influenced by the chaotropicity of the counteranion type and concentration in the mobile phase. Varying the amount of organic modifier in the mobile phase changed retention, but did not affect the separation factor. It was found, that the separation was enthalpy driven, so the best separation was achieved using 2% (v/v) TFA in 95/5 (v/v) water-/methanol as mobile phase with a column temperature at 5°C. It was also found, that air is a better carrier gas than nitrogen and the best S/N was

achieved when air was set at 1.5 psi. The optimum operation condition for the drift tube was found to be at 115°C. The mobile phase flow rate could not exceed 0.5 mL/min, in order to ensure the complete vaporization of the mobile phase.

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