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Enantioselective ion-exclusion chromatography on teicoplanin aglycone and (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid stationary phases

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Abstract

Ion-exclusion chromatography is a well established technique for the analysis of achiral ionic species, but it has rarely been applied to chiral analytes. In this paper enantioselective ion-exclusion separations were developed on two commercially available HPLC phases: Chirobiotic TAG, based on teicoplanin aglycone, and Opticrown RCA (+), based on (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid. Chirobiotic TAG columns have a carboxylic acid group on the chiral ligand, which can be partially ionized to exclude anionic analytes by ionic repulsion. Under acidic conditions Opticrown columns have a cationic sublayer generated from the aminopropyl base silica that excludes cationic analytes. Both columns demonstrate a large dependence of efficiency on flow-rate, with the highest efficiencies at 0.1 ml/min on a 4.6 mm inner diameter column.

Keywords: Enantiomer separation; Chiral stationary phases, LC; Ion exclusion; Teicoplanin aglycone; (+)-(18-Crown-6)-2,3,11,12-tetracarboxylic acid

1. Introduction

In the enantioselective separations of dansyl amino acids on a Chirobiotic TAG (Astec) column we observed unusually small elution volumes of the enantiomers. Typically on a 250×4.6 mm packed column the void volume (elution volume of an unretained neutral analyte) is between 2.4 and 3.0 ml. In our study we obtained partial or complete enantioselective resolution with the first peak eluting as early as 2.022 ml. Further study and optimization indicated that ion exclusion played a significant role in this separation. As with ion-exclusion chromatography, we found that this mode of separation has general character and can be applied to either anions or cations on different chiral stationary phases that bear the same charge on their surface as the analyte. We call this phenomenon enantioselective ion-exclusion chromatography, which reflects enantioselectivity that is observed in the ion-exclusion mode of chromatography.

Ion-exclusion chromatography was first introduced by Wheaton and Bauman in 1953 [1] and has been routinely applied to acids, amines, inorganic anions and cations, alcohols, and sugars. Fritz [2] and Glod

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[3] have written extensive reviews of these applications. In ion-exclusion chromatography, ion-exchange resins are used to separate ions that have the same charge as the ion-exchange resin. Tanaka et al. [4] found that the elution volume of an acidic solute was dependent primarily on the first acid dissociation constant, pK_{a1}, of the solute. This dependence between the sample elution volume and its pK_{a1} value could be explained by the magnitude of the charge on the solute. That is, all solutes which were fully ionized at the eluent pH were unretained by virtue of their repulsion by the anionic functional groups of the stationary phase and were eluted at the column void volume v_o. Neutral solutes were all co-eluted at a volume equal to the sum of the void and inner volumes of the column, v_r, since they were able to partition freely between the eluent and the inner volume, the occluded liquid trapped within the pores of the stationary phase. This v_r is the void that is commonly referred to in adsorptive modes of chromatography. This description of ion-exclusion chromatography assumes that there are no adsorptive interactions between the carboxylic acid and the resin surface. However, hydrophobic and steric effects have been described in the literature [5–14], and these effects have been both inhibited and enhanced to improve resolution. By varying the spacer molecule connecting the sulfonate group to silica, Klampfl et al. [9] were able to manipulate the contribution of hydrophobic interactions to the retention of carboxylic acids, which allowed an increase of separation efficiency and selectivity in ion-exclusion mode.

The application of ion-exclusion chromatography to chiral analytes has been limited. Glod and Perez [12] enantioselectively separated methylphenobarbital on an ion-exclusion column by adding β-cyclodextrin to the mobile phase. Gahm and Stoianov [15] observed the enantioselective separation of 6,8-dichloro-2-(trifluoromethyl)-2*H*-1-benzopyran-3-carboxylic acid (Fig. 1) before the column void, v_r, on a Chirobiotic T column (Astec) with a mobile phase consisting of methanol/acetic acid/triethylamine (1000/0.1/0.05, v/v). Chirobiotic T, based on teicoplanin covalently bonded to silica, was developed by Armstrong et al. and is a versatile chiral phase with multiple retention mechanisms that can be used in reversed-phase, normal-phase, and polar organic mode separations [16]. In the above mobile phase, the teicoplanin ligand contains both a partially ionized carboxylic acid, which provides the anionic exclusion required for ion-exclusion chromatography, and also additional enantioselective interaction sites that enable the chiral separation.

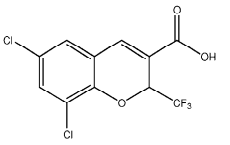

Fig. 1. 6,8-Dichloro-2-(trifluoromethyl)-2*H*-1-benzopyran-3-carboxylic acid.

In this paper we present enantioselective ion-exclusion separations for anions (dansyl amino acids) and cations (amino acids). Fig. 2 shows the general structure of dansyl amino acids. The anion separations use a Chirobiotic TAG column based on teicoplanin aglycone (Fig. 3) bonded to silica. Teicoplanin aglycone is obtained by the removal of the carbohydrates from teicoplanin under acidic conditions [17]. The polar organic mode with mobile

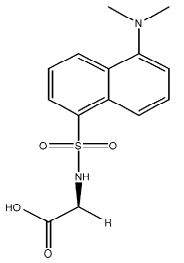

Fig. 2. The general structure of dansyl amino acids.

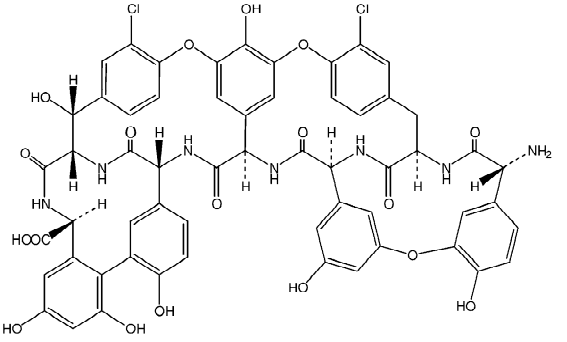

Fig. 3. Structure of TAG (teicoplanin aglycone) ligand.

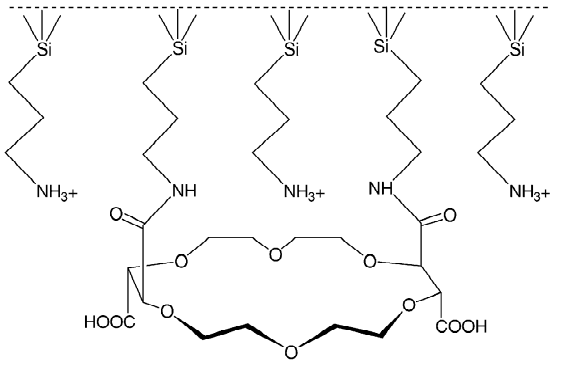

Fig. 4. Structure of the Opticrown RCA (+) phase.

phases consisting of methanol, acetic acid, and triethylamine was used to separate dansyl amino acids. The chiral ligand provides both a carboxylate anion, which creates anionic exclusion, and also provides the enantioselective interactions to enable the chiral separation. These interactions are due to any of the possible interactions provided by the chiral ligand: pi–pi complexation, hydrogen bonding, inclusion, dipole stacking, steric, and ionic binding.

For the enantioselective ion-exclusion separations of cations (amino acids), Opticrown RCA (+) columns (USmac) were used in a mobile phase of 10 m*M* trifluoroacetic acid in 80/20 methanol/water (v/v). This phase, based on technology developed by Hyun et al. [18], consists of (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid covalently bonded to aminopropyl silica (Fig. 4). Under acidic conditions these columns have an achiral cationic sublayer generated by the residual aminopropyl groups. This sublayer creates cationic exclusion for the primary amine of the amino acids, but does not prevent the chiral crown ether complexation with the same primary amine.

2. Experimental

The amino acids, dansyl-amino acids, trifluoroacetic acid, and triethylamine were purchased from Sigma–Aldrich (Milwaukee, WI, USA). Acetic acid and acetonitrile were obtained from EM Science (Gibbstown, NJ, USA) and methanol was obtained from Burdick and Jackson (Muskegon, MI, USA). Water was filtered through a Milli-Q system from Millipore (Bedford, MA, USA). Chirobiotic TAG, T and V columns were obtained from Astec (Whippany, NJ, USA) and measured 250×4.6 mm. Opticrown RCA (+) columns were obtained from USmac (Thousand Oaks, CA, USA) and measured 150×4.6 mm. All separations were confirmed on multiple columns. The HPLC system (Hewlett-Packard, Palo Alto, CA, USA) consisted of a Hewlett-Packard Series 1100 HPLC system with quaternary pump, autosampler, degasser, diode array detector, mass spectrometric detector, and column oven with mobile phase preheater. Mass spectrometric parameters were drying gas 13 l/min, nebulizer pressure 60 p.s.i.g., drying gas 350 °C, fragmentor 100 V, and capillary voltage 4000 V. All instrumentation was calibrated and maintained by an in-house metrology department. All samples were prepared daily by dissolving in the appropriate mobile phase at 0.5 mg/ml for racemates and 0.25 mg/ml for single isomers. Efficiency and resolution calculations were performed by the Hewlett-Packard Chemstation™ software by using peak width at half height for plate count. Tabulated data are representative of duplicate injections. Relative standard deviations (RSDs) and height equivalent of a theoretical plate (HETP) versus flow-rate curves were generated from triplicate injections.

3. Results and discussion

3.1. Enantioselective ion-exclusion separations of dansyl-amino acids on Chirobiotic TAG (teicoplanin aglycone)

The chromatograms in Fig. 5 show the best separations obtained for each dansyl amino acid, and Table 1 lists the mobile phase, column temperature, retention times, resolution and selectivity. As can be seen the best separations were obtained for dns-methionine, dns-norvaline, dns-phenylalanine, and dns-serine with the other dansyl amino acids only marginally separated. Dns-aspartic acid and dns-glutamic acid were not successfully separated with good peak shape and were not included. RSD between triplicate injections on a single column for dns-DL-met were 0.04% for peak 1 and 0.15% for peak 2. RSD between three columns of two different

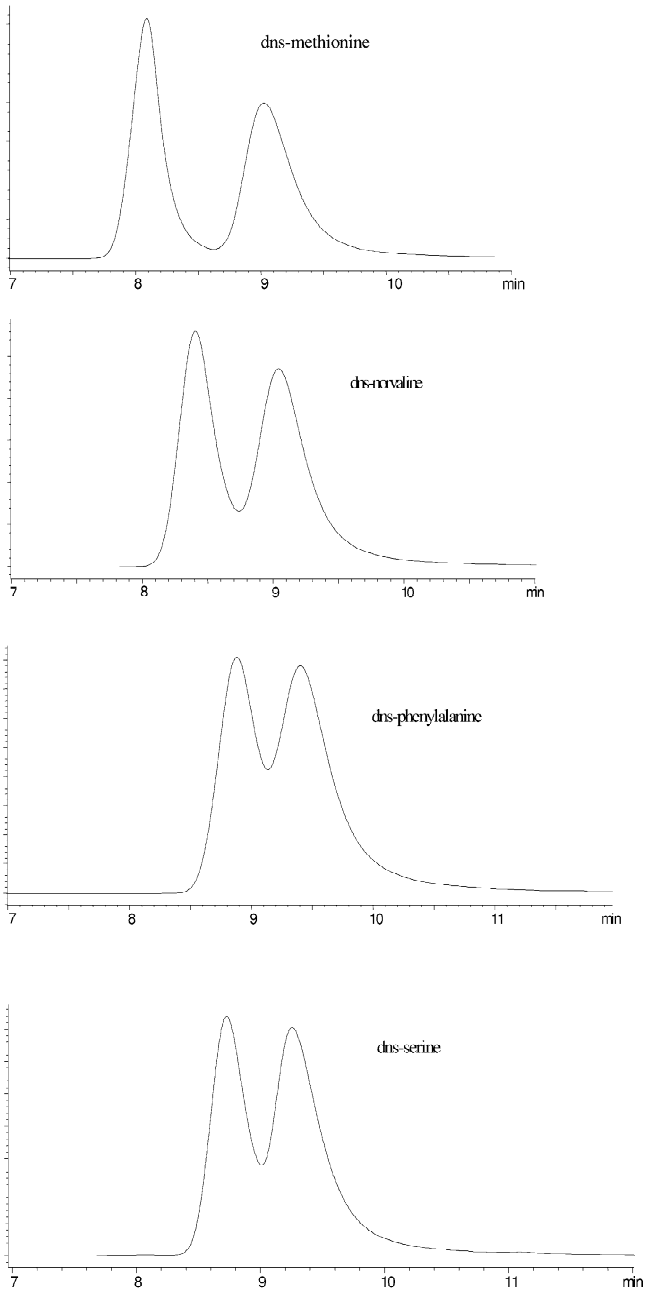

Fig. 5. Representative chromatograms for the enantioselective ion-exclusion separations of dansyl amino acids on a Chirobiotic TAG column at a flow-rate of 0.25 ml/min and λ=254 nm. *Y*-axis in minutes.

lots (confirmed by the vendor) were 0.26% for peak 1 and 3.82% for peak 2.

The chromatograms presented were generated at 0.25 ml/min on a 4.6 mm inner diameter column. These separations show a large dependence of efficiency on flow-rate as shown in Fig. 6 for dns-L-methionine, the first isomer eluted for dns-DL-methionine. Efficiency increases continually as the

Table 1
Separation data for the chromatograms in Fig. 1

Analyte	Mobile phase	Column temp., °C	Elution volumes (ml)	Selectivity ($\alpha = k'2/k'1$)	Resolution[a]
Dns-α-amino-n-butyric acid	A	25	2.136/2.212	1.187	–
Dns-leucine	A	10	2.106/2.169	1.167	–
Dns-methionine	B	10	2.022/2.256	1.793	1.64
Dns-norvaline	A	10	2.101/2.260	1.425	1.06
Dns-phenylalanine	A	10	2.220/2.351	1.265	0.64
Dns-serine	A	45	2.182/2.314	1.289	0.81
Dns-threonine	A	25	2.106/2.182	1.201	–
Dns-tryptophan	A	25	2.387/2.454	1.103	–
Dns-valine	A	10	2.096/2.169	1.200	–

Chirobiotic TAG column at 0.25 ml/min and $\lambda = 254$ nm. Mobile phase A: methanol/acetonitrile/acetic acid/triethylamine (1000/0/0.05/0.15, v/v); mobile phase B: methanol/acetonitrile/acetic acid/triethylamine (950/50/0.05/0.15, v/v).

[a] Chemstation™ software calculated plate count using peak width at half height. For accuracy, resolution is listed only for analytes that were resolved at half height.

flow-rate is lowered from 2 to 0.1 ml/min. The improvement of efficiency with reduced flow-rate on a chiral stationary phase has been studied by Marle et al. [19] and attributed to slow adsorption–desorption kinetics. Rizzi demonstrated a similar flow-rate dependence for ligand exchange chromatography of amino acids with efficiency increasing down to 0.15 ml/min on a 4 mm inner diameter column [20]. While 0.1 ml/min provides the highest efficiency in our examples, we chose 0.25 ml/min for faster analysis times, 8 versus 20 min.

In order to establish ion exclusion in this system, we needed to measure v_r, the elution volume of a non-retained and non-excluded analyte. Ng et al. demonstrated the use of methanol for this purpose on a strong cation-exchange resin [11]. But in chiral separations, where the chiral selector has multiple interactions with an analyte, it is difficult to find probes that have no interaction with the stationary phase. We determined a v_r of 2.70 ml by using a mobile phase of 50% (acetonitrile/acetic acid/triethylamine 1000/0.05/0.15, v/v) and 50% (methanol/acetic acid/triethylamine 1000/0.05/0.15, v/v). The sample probes used were toluene dissolved in the mobile phase, neat methanol, and neat acetonitrile. All three probes gave v_r within 0.1 ml of 2.70 ml. If mobile phases of 100/0 and 0/100 of the above two mobile phase components were used, larger v_r values were obtained without such close agreement.

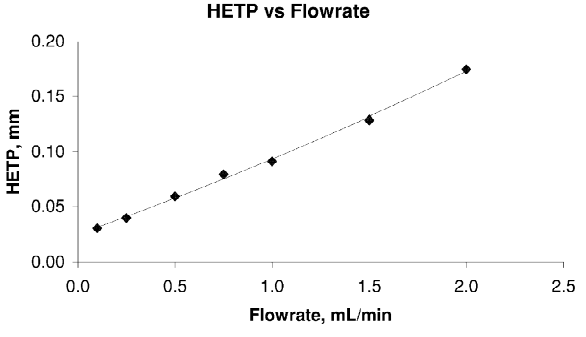

Fig. 6. HETP versus flow-rate for dns-L-methionine on Chirobiotic TAG, 250×4.6 mm. HPLC conditions listed in Table 1.

A larger effect on elution volumes was seen when the buffer concentration was changed. The effect of buffer concentration for selected dansyl amino acids is shown in Table 2. Elution volumes decreased as buffer concentration also decreased. At the initial concentration, which is typical of polar organic mode separations [21], all peaks retain after v_r. Reducing the concentration to one-fifth of the initial concentration resulted in peak 1 for all three dansyl amino acids eluting before v_r. A further reduction to one-tenth of the initial concentration resulted in all peaks eluting before v_r except for peak 2 of dns-DL-methionine. Eliminating the buffer entirely resulted in the smallest elution volumes of all with dns-DL-methionine eluting at 1.727 ml, however enantioselectivity was lost. We attribute this to a reduction of ionic strength with reduction of buffer concen-

Table 2
Effect of buffer concentration on separation

Mobile phase: methanol/acetic acid/triethylamine (v/v)	Dns-DL-α-amino-n-butyric acid elution volumes (ml)	Dns-DL-methionine elution volumes (ml)	Dns-DL-phenylalanine elution volumes (ml)
1000/0.5/1.5	3.884/4.303	3.742/5.461	3.801/4.298
1000/0.1/0.3	2.674/2.847	2.643/3.483	2.693/2.934
1000/0.05/0.15	2.426/2.426	2.306/2.818	2.209/2.450

Chirobiotic TAG column with v_r of 2.70 min, flow-rate of 1 ml/min and λ=254 nm.

tration, and this effect has been reported by Crone [22] for the ion-exclusion separation of amines.

In order to calculate capacity factors we used the elution volume of dns-DL-methionine in methanol, 1.727 ml, as v_o. This value is in close agreement with those obtained with dns-DL-α-amino-*n*-butyric acid and dns-DL-phenylalanine in methanol, 1.782 and 1.746 ml, respectively. As mentioned earlier in the discussion about determining v_r, it is difficult to determine v_o due to the multiple types of interaction available on this chiral stationary phase. We tried other probes which would be either fully dissociated or expected to have little or no interaction with the chiral ligand in methanol, such as hydrochloric acid, maleic acid, trifluoroacetic acid and acetic acid. All gave larger elution volumes than dns-DL-methionine in a methanol mobile phase.

The acetic acid concentration in the mobile phase was also studied (Table 3). While the triethylamine concentration was kept constant, as the volume of acetic acid increased from 0.05 to 0.30 ml, the capacity factors increased. A peak with k' greater than 0.680 is no longer an excluded peak and at 0.30 ml acetic acid no dansyl amino acids are excluded. An increase to 0.45 ml shows a reduction in elution volume and all peaks are again excluded. As can be seen in Table 3, 0.05 ml acetic acid is the optimal acetic acid level for generating the best resolution for dns-methionine in the ion-exclusion mode.

Column temperature plays a role in enantioselective separations and Table 4 shows the effect of temperature on selectivity and resolution for selected dansyl amino acids. Except for dns-serine at 45 °C, higher resolution was obtained at column temperatures of 10 and 25 °C. In all cases, increasing the temperature resulted in reduced elution volumes, but the effect on resolution differed for each compound.

The effects of adding up to 20% acetonitrile to the mobile phase were also studied. In all cases adding acetonitrile slightly reduced elution volumes and except for dns-methionine, adding acetonitrile reduced resolution. For dns-methionine, the second

Table 3
The effect of acetic acid concentration

	1000/0.05/0.15	1000/0.15/0.15	1000/0.30/0.15	1000/0.45/0.15
Dns-α-amino-*n*-butyric acid	$k'1$=0.244 $k'2$=0.244 α=1.000 R_s=0.00	$k'1$=0.515 $k'2$=0.515 α=1.000 R_s=0.00	$k'1$=0.622 $k'2$=0.622 α=1.000 R_s=0.00	$k'1$=0.540 $k'2$=0.540 α=1.000 R_s=0.00
Dns-methionine	$k'1$=0.208 $k'2$=0.393 α=1.891 R_s=1.27	$k'1$=0.453 $k'2$=0.752 α=1.661 R_s=1.44	$k'1$=0.607 $k'2$=0.944 α=1.544 R_s=1.46	$k'1$=0.276 $k'2$=0.436 α=1.579 R_s=0.89
Dns-phenylalanine	$k'1$=0.256 $k'2$=0.256 α=1.000 R_s=0.00	$k'1$=0.537 $k'2$=0.537 α=1.000 R_s=0.00	$k'1$=0.701 $k'2$=0.701 α=1.000 R_s=0.00	$k'1$=0.299 $k'2$=0.299 α=1.000 R_s=0.00

Mobile phase was methanol/acetic acid/triethylamine (1000/x/0.15, v/v) at 1 ml/min and λ=254 nm.

Table 4
The effect of column temperature on selectivity and resolution

	10 °C		25 °C		45 °C	
	Selectivity	Resolution	Selectivity	Resolution	Selectivity	Resolution
Dns-methionine	1.793	1.64	1.746	1.59	1.687	1.43
Dns-norvaline	1.425	1.06	1.384	0.95	1.330	0.71
Dns-phenylalanine	1.265	0.64	1.237	–	1.207	–
Dns-serine	1.331	0.73	1.316	0.80	1.289	0.81

Mobile phase conditions are listed in Table 1 for each compound.

isomer eluted close to v_r, which reduced this peak's efficiency. Adding acetonitrile reduced this peaks elution volume and increased its efficiency, which in turn increased resolution.

Other Chirobiotic columns were tested as well. Chirobiotic T also produced enantioselective ion-exclusion separations, but resolution was less than on Chirobiotic TAG. This is possibly due to the carbohydrates on teicoplanin interfering with the inclusion of the analytes within the cavities present in the ligand [17]. Chirobiotic V (Astec), based on vancomycin, provided ion exclusion, but no enantioselectivity was obtained for dansyl amino acids.

3.2. Enantioselective ion-exclusion separations of amino acids on an Opticrown RCA (+)

Another chiral stationary phase demonstrates enantioselective ion-exclusion chromatography for chiral cations. Fig. 7 shows the enantioselective ion-exclusion separation of selected amino acids on an Opticrown RCA (+) column. Both peaks for arginine and valine and the first peak in the remaining chromatograms elute in ion-exclusion mode, i.e. before v_r (1.453 ml).

Table 5 lists the separation data for 19 amino acids. Peaks with retention volumes greater than 1.453 ml elute after v_r. All separations were done with UV and mass spectrometric detection with a positive electrospray source in series and the correct masses were confirmed in all cases.

As can be seen from Table 5, arginine and valine showed partial resolution in the ion-exclusion mode. The first peaks of histidine, leucine, phenylalanine, tryptophan and tyrosine eluted in the ion-exclusion mode while the second peaks showed adsorptive retention. The remaining amino acids either did not show resolution or both peaks showed adsorptive retention. RSD between triplicate injections of DL-arginine on a single column were 0.07% for peak 1 and 0.11% for peak 2. RSD between triplicate injections on three columns of two different lots (+ and − isomer) were 4.59% for peak 1 and 6.93% for peak 2. The Opticrown SCA(−) column had slightly higher elution volumes which generated larger RSD values between columns than for Chirobiotic TAG.

As for Chirobiotic TAG, the highest efficiencies were generated at very low flow-rates for a 4.6 mm inner diameter column. Fig. 8 shows the plot of HETP versus flow-rate for L-arginine, the first eluted isomer of DL-arginine. As for the Chirobiotic TAG column efficiency continues to increase as flow-rate is decreased from 2 to 0.1 ml/min, so we chose 0.25 ml/min as a compromise between efficiency and analysis time.

Hyun et al. also applied columns based on (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid to amino acids using a mobile phase of 10 m*M* sulfuric acid in 80/20 methanol/water and obtained retentive separations in all cases [18]. The elution order of the amino acid isomers was identical in both cases except for serine.

The determination of v_r for the Opticrown column has the same difficulty as for the Chirobiotic TAG. A similar procedure was employed by injecting toluene dissolved in mobile phase, neat acetonitrile, and neat methanol in a mobile phase of 10 m*M* trifluoroacetic acid in 80/20 methanol/water (v/v). Toluene eluted at 1.566 ml, acetonitrile at 1.453 ml, and methanol at 1.855 ml. If the acetonitrile in the mobile phase is replaced with methanol larger elution volumes result. We chose 1.453 as v_r. v_o was determined by injecting tertiary amines in a mobile phase of 10 m*M* trifluoroacetic acid in 80/20 methanol/water. Tertiary

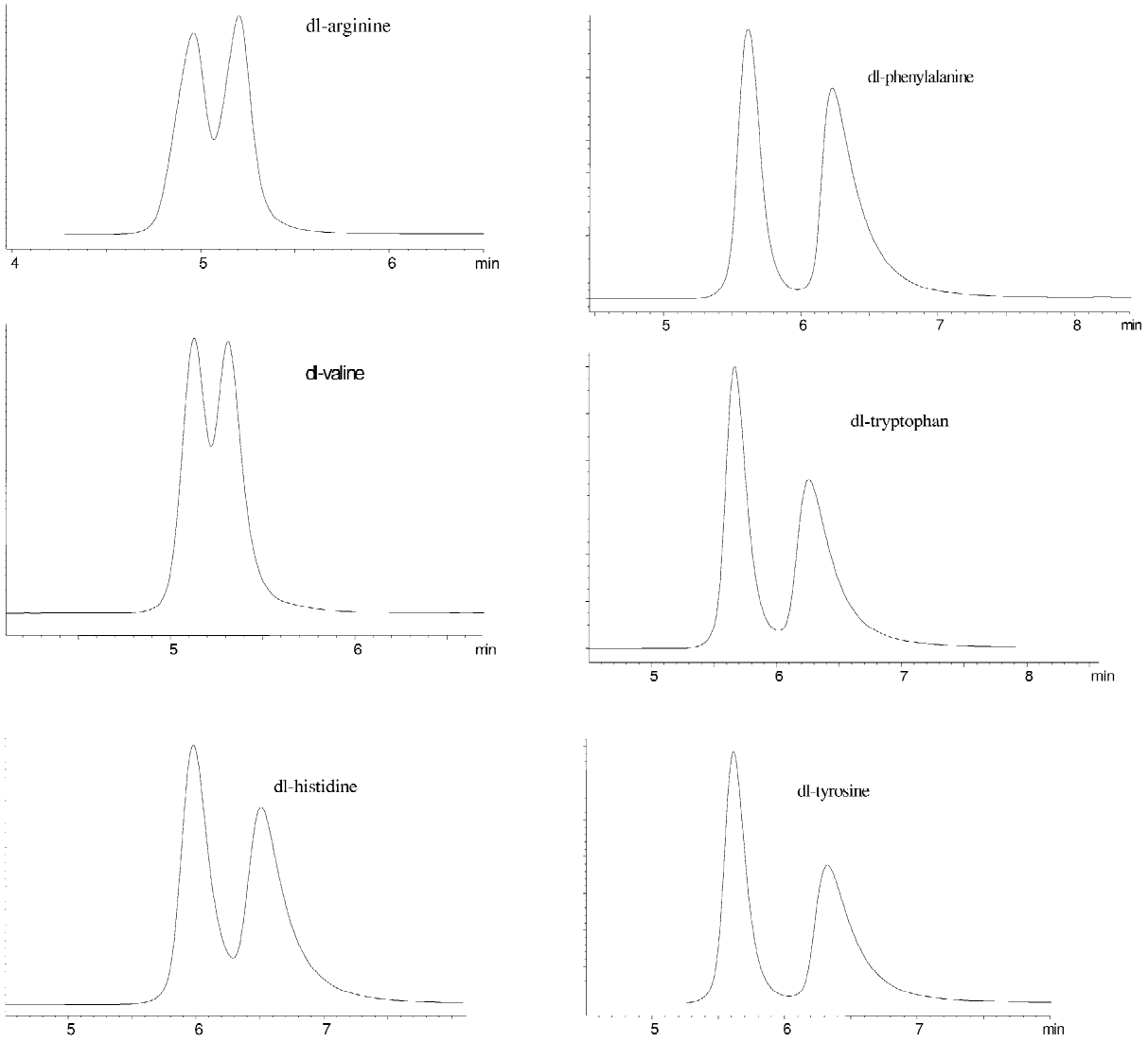

Fig. 7. Enantioselective ion-exclusion separations of amino acids on Opticrown RCA (+) with a mobile phase of 10 m*M* trifluoroacetic acid in 80/20 methanol/water at 0.25 ml/min with UV detection at 210 nm. *Y*-axis in minutes. Both peaks are arginine and valine elute in ion-exclusion mode. The first peak in the remaining chromatograms elute in ion-exclusion mode.

amines have not been shown to retain on crown ether stationary phases, and at the low pH of this mobile phase little or no interaction with the carboxylic acid groups on the chiral crown ether is expected. We obtained 1.117 ml for chloroquin, 1.210 ml for trimipramine, 1.212 ml for bupivacaine, and 1.218 ml for trihexyphenidyl. We chose the smallest value of 1.117 ml as v_o.

The effect of trifluoroacetic acid concentration on tryptophan elution volumes was studied (Table 6). As was shown in Table 4 for acetic acid and the Chirobiotic TAG column, trifluoroacetic acid concentration has a significant effect on elution volumes. The smallest elution volumes were obtained with 10 m*M* trifluoroacetic acid and the first peak of tryptophan eluted in ion-exclusion mode. In all other cases both peaks eluted in adsorptive mode after v_r. The highest adsorption was obtained for 1 m*M*

Table 5
Separation data of amino acids on Opticrown RCA (+) column, 150×4.6 mm

Analyte	Elution volumes (ml)	Selectivity ($\alpha = k'2/k'1$)	Resolution[a]	First eluted isomer
Alanine	1.527/1.641	1.279	1.24	l
Arginine	1.241/1.301	1.489	0.76	l
Asparagine	1.550/1.639	1.205	0.76	l
Aspartic acid	2.105/2.356	1.254	–	l
Cysteine	1.695/1.803	1.188	–	l
Glutamic acid	1.576/1.871	1.643	1.90	l
Glutamine	1.514/1.717	1.513	1.76	l
Histidine	1.408/1.509	1.349	1.12	l
Isoleucine	1.285/1.285	1.000	–	l
Leucine	1.351/1.492	1.605	1.77	l
Lysine	1.847/1.967	1.165	–	l
Methionine	1.512/1.768	1.649	2.13	l
Phenylalanine	1.404/1.558	1.535	1.62	l
Proline	1.311/1.311	1.000	–	l
Serine	1.620/1.747	1.252	0.83	d
Threonine	1.324/1.324	1.000	–	l
Tryptophan	1.416/1.565	1.496	1.45	l
Tyrosine	1.404/1.581	1.617	1.76	l
Valine	1.282/1.328	1.278	–	l

v_o=1.117 ml and v_r=1.453 ml. Mobile phase was 10 m*M* trifluoroacetic acid in 80/20 methanol/water (v/v) with flow-rate of 0.25 ml/min at λ=210 nm.

[a] Chemstation™ software calculated plate count using peak width at half height. For accuracy, resolution is listed only for analytes that were resolved at half height.

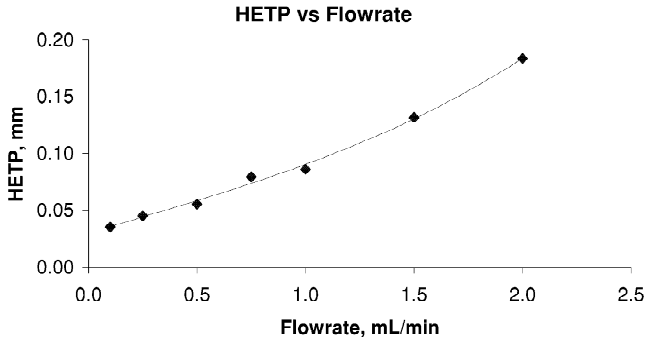

Fig. 8. HETP versus flow-rate for L-arginine on Opticrown RCA (+), 150×4.6 mm. HPLC conditions listed in Table 5.

Table 6
The effect of trifluoroacetic acid concentration in 80/20 methanol/water (v/v)

	1 m*M* TFA	5 m*M* TFA	10 m*M* TFA	20 m*M* TFA
Tryptophan	$k'1$=0.806	$k'1$=0.302	$k'1$=0.284	$k'1$=0.338
	$k'2$=1.461	$k'2$=0.474	$k'2$=0.406	$k'2$=0.422
	α=1.813	α=1.470	α=1.429	α=1.246
	R_s=2.29	R_s=1.27	R_s–	R_s–

Opticrown RCA (+), 150×4.6 mm at λ=210 nm.

trifluoroacetic acid. This may be because 1 m*M* TFA has the highest pH (3.16 vs. 2.46 for 10 m*M* TFA, measured in water) resulting in the higher ionic attraction between the carboxylate on the amino acid and the aminopropyl sublayer on the stationary phase. The effect of methanol concentration in the mobile phase was also studied, and only a slight decrease in elution volume with increasing methanol was observed.

4. Conclusion

In this paper we presented enantioselective ion-exclusion separations of cations and anions on two commercially available chiral stationary phases. In these separations the role of buffer and acid concentration played a dominant role in creating ion exclusion. These separations showed the highest efficiencies at very low flow-rates, 0.1 ml/min on a 4.6 mm inner diameter column.

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