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Supercritical fluid chromatography comparison of the poly(*trans*-1,2-cyclohexanediyl-bis acrylamide) (P-CAP) column with several derivatized polysaccharide-based stationary phases☆

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ABSTRACT

The poly(*trans*-1,2-cyclohexanediyl-bis acrylamide) (P-CAP) column has so far been primarily used with normal phase and polar organic mobile phase chromatography. Its use in supercritical fluid chromatography (SFC) was investigated via the analysis of 40 commercial and 100 proprietary compounds using a 12-min gradient with methanol as a modifier. Results were then compared against those obtained from the popular derivatized polysaccharide-based chiral stationary phases (CSPs) such as Chiralpak AD-H and Chiralpak AS-H as well as Chiralcel OD-H and Chiralcel OJ-H columns. P-CAP demonstrated separation of 25% of the 140 total compounds, while each of the derivatized polysaccharide-based CSPs separated at least 46%. A study that compared the loading of 1,1′-bi-2-naphthol with P-CAP and Chiralpak AS columns indicated a similar trend in resolution vs. amount injected, though AS appeared capable of allowing a greater loading of material. The P-CAP column was found to be beneficial in the separation of a complex mixture of enantiomers and achiral impurities, where the derivatized polysaccharide-based columns did not show as desirable of a separation. A key advantage of this type of chiral stationary phase is the fact that it is available in both enantiomeric forms, allowing manipulation of elution order of enantiomers, which is especially helpful for preparative applications. P-CAP also demonstrated that it could resolve an achiral impurity from the desired compound in a different mixture, while the same impurity co-eluted on the Chiralpak AD-H column. Overall, the synthetic polymer-based P-CAP showed less chiral discrimination power compared to the derivatized polysaccharide-based CSPs under the conditions explored in this study.

1. Introduction

Separation of chiral pharmaceutical compounds is an important chromatographic field [1,2], since chirality is an important issue in drug development [3–6]. The activity of each enantiomer must be investigated due to potential differences in activity and toxicity [6–8]. New chiral stationary phases provide potential sources of improved or complementary chiral separations. Therefore, they must be carefully evaluated to understand their use in the chiral separation work-flow of a laboratory. The poly(*trans*-1,2-cyclohexanediyl-bis acrylamide) (P-CAP) column is available in *R*,*R* and *S*,*S* forms, which allows the control of elution order of enantiomers. It has been previously evaluated via normal phase and polar organic modes of chromatography [9], and recently, it has been utilized with supercritical fluid chromatography (SFC) [10].

The use of P-CAP in SFC is of interest, since SFC is a powerful technique utilized for chiral separations, due to its inherent advantages such as high diffusivity and low viscosity which typically allow short run times and higher peak efficiency [1,4,11–19]. To evaluate the P-CAP column by SFC, a total of 40 commercial compounds and 100 proprietary compounds were analyzed. The compounds were then analyzed by the derivatized polysaccharide-based chiral stationary phases (CSPs) (the amylose-based Chiralpak AD-H and Chiralpak AS-H columns as well as the cellulose-based Chiralcel OD-H and Chiralcel OJ-H columns) via the same 12-min gradient SFC screening method. Chiralpak and Chiralcel columns were chosen due to their extensive use in industry for chiral separations [1,20,21].

During this investigation, the P-CAP column was applied to solve actual separation/purification problems. A difficult preparative separation was facilitated by the application of the P-CAP column. P-CAP was used to separate a complex mixture of

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enantiomers and achiral impurities (mixture A) and showed a clear advantage over the other four columns in this particular case. All three components (two enantiomers and a major achiral impurity) were well separated in less than 3 min, analytically. In addition, the P-CAP column simplified the determination of enantiomeric peak pairs by providing the ability to switch peak elution order of the enantiomers without affecting the retention time of the major impurity. Overall, the results of this study have increased understanding of the potential of the P-CAP column and its suggested role in the current work-flow.

2. Experimental

2.1. Materials

The SFC-grade carbon dioxide was obtained from BOC Gases (Murray Hill, NJ, USA). Methanol (MeOH) and acetonitrile (ACN) were HPLC-grade from Mallinckrodt Baker (Muskegon, MI, USA). Diethylamine (DEA) was of analytical grade and from Sigma–Aldrich (St. Louis, MO, USA).

All of the 40 commercial compounds were purchased from Sigma–Aldrich (St. Louis, MO, USA). The 100 proprietary compounds were synthesized within Amgen Inc. and chosen from a variety of different projects to ensure diversity.

Mixtures A and B are proprietary reaction products prepared within Amgen, Inc. Mixture A was comprised of two enantiomers with major and minor achiral impurities. Mixture B was primarily comprised of a major enantiomer with several minor impurities, including the opposite enantiomer.

2.2. Analytical SFC instrumentation

The analytical SFC instrument was a Berger SFC unit (Thar Technologies, Pittsburgh, PA, USA) with an FCM1200 flow control module, a dual pump control module, a TCM2100 thermal column module (temperature is controlled from 7 to 150 °C), a column selection valve capable of switching between six columns, and a solvent control valve for up to six modifiers to be selected. The SFC was equipped with an Agilent 1100 photodiode array detector with a high-pressure flow cell (Agilent Technologies, Palo Alto, CA, USA). The autosampler/injector was a CTC LC Mini PAL from Leap Technologies (Carrboro, NC, USA). A Waters (Milford, MA, USA) ZQ benchtop single quadrapole mass spectrometer with an atmospheric pressure chemical ionization (APCI) source was coupled to the SFC. The software used in the analyses was Berger MassWare™ v. 4.01 and MassLynx™ v. 4.0 SP1.

2.3. Preparative instrumentation

The preparative SFC was a Berger Multigram™ II from Thar Technologies (Pittsburgh, PA, USA). Components included the Separator Control Module (SCM)-2500, Electronics Control Module (ECM)-2500, CO_2 solvent delivery module, modifier solvent delivery module, direct expansion probe chiller, ventilated collection cabinet, UV variable wavelength detector, and a waste containment vessel. The injector was a Modular Digital Pump (Model XL3000) from Cavro Scientific Instruments Inc. (Sunnyvale, CA, USA). Software used in the purification was Berger SFC ProNTo™ v. 1.5.305.15.

2.4. Chiral columns

The analytical chiral packed columns included the Supelco P-CAP column (Bellefonte, PA, USA) as well as Chiral Technologies (West Chester, PA, USA) Chiralpak AD-H and Chiralpak AS-H and Chiralcel OD-H and Chiralcel OJ-H. These columns are referred to as P-CAP, AD-H, AS-H, OD-H, and OJ-H throughout the paper. Dimensions of the columns were 150 mm × 4.6 mm I.D. with 5 μm particle size.

For preparative SFC, the chiral columns consisted of P-CAP and Chiralpak AD-H. Both columns had dimensions of 250 mm × 21 mm with 5 μm particle size. In addition, P-CAP and Chiralpak AS columns (250 mm × 21 mm with 10 μm particle size) were used. Columns were purchased from Supelco (Bellefonte, PA, USA) and Chiral Technologies (West Chester, PA, USA). The preparative columns are referred to hereafter as prep-P-CAP, prep-AD-H, prep-P-CAP$_{10\mu m}$ (10 μm particle size column), and prep-AS$_{10\mu m}$ (10 μm particle size column).

2.5. Analytical SFC screening method and calculations

The screening method utilized a gradient with a mobile phase that consisted of liquid CO_2 (A) and organic modifier (B). Methanol (with 0.2% DEA as needed) was used for B. The gradient started at 7.0% B and was held for 2 min. Next, B was increased to 50.0% at a rate of 7.0%/min. After reaching 50.0%, this was held for 2 min before returning to 7.0% B at a rate of 99.9%/min and then held for 2 min. The flow rate was 4.0 mL/min. Total run time was 12 min. Column oven and nozzle temperature were 35 °C, and the outlet pressure was 100 bar. A total of 40 commercial compounds and 100 proprietary compounds were screened.

Retention times were obtained from the data generated via MassLynx™ v. 4.0 SP1. Dead times (t_0) for the commercial compounds were estimated by using the retention time of the peak that resulted from the change in refractive index from the injection solvent. The retention times for Amgen compounds were obtained from the APCI (+) extracted ion chromatograms (EICs). Mass spectrometry was utilized due to its ability to allow "sample pooling" [3]. At least eight samples of different molecular weights were combined as one solution for analysis, allowing a dramatic reduction in injection numbers and overall analysis time. Dead time was estimated by using the retention time of the first disturbance in the total ion chromatograms (TICs) which corresponded with the disturbance also found in the UV chromatograms.

Since a gradient was utilized, the common chromatographic calculations/terms of retention factor and selectivity were not used. Instead, the terms used to describe chromatographic phenomena of the gradient data are relative retention times (rRT) and delta retention times (Δ_{RT}). The rRT was calculated by subtracting t_0 from the retention time. Calculation of Δ_{RT} was performed by taking the difference between the rRT values of two peaks.

A Δ_{RT} value of 0.05 min or greater was considered sufficient for chiral discrimination. Though a Δ_{RT} time greater than or equal to 0.05 min would not necessarily guarantee a successful scale-up, it did show that some separation took place; a successful scale-up may be possible with further method development.

Retention factors (k') and selectivity values (α) were used for the isocratic loading study. The k' values were calculated using the traditional chromatographic equation ($k' = (t_R - t_0/t_0)$), and α was calculated by taking the ratio of the retention factor of the longer retaining peak (k'_2) to the retention factor of the less retained peak (k'_1). Resolution was calculated using the equation of $R = 2(t_2 - t_1)/(w_1 + w_2)$, where t is retention time, and w is peak width at base height.

2.6. Preparative analysis conditions for the loading study of 1,1′-bi-2-naphthol

The mobile phase consisted of liquid CO_2 and MeOH. Both methods (for prep-P-CAP$_{10\mu m}$ and prep-AS$_{10\mu m}$) were isocratic with a

Table 1
Neutral compound (non-nitrogen containing) structures and the corresponding relative retention time (rRT_1) of peak 1 and delta retention times (Δ_{RT}) for P-CAP, AD-H, OD-H, OJ-H, and AS-H columns

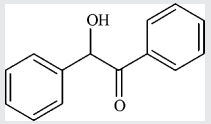
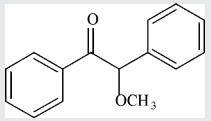
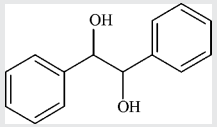
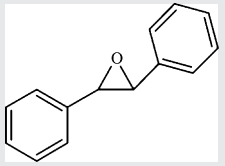
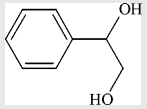
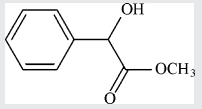
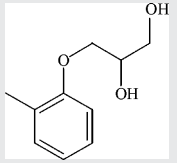
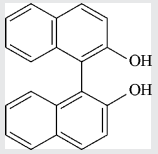
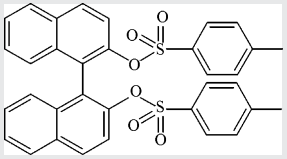
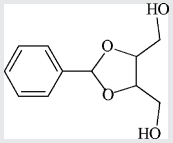
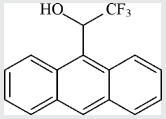
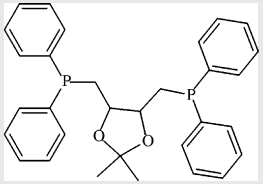
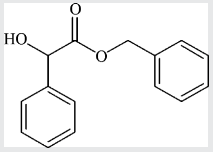
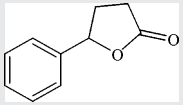
No.	Compound	PCAP		AD-H		OD-H		OJ-H		AS-H	
		Neutral compounds (Non-nitrogen containing)									
		rRT_1 (min)	Δ_{RT} (min)	rRT_1 (min)	Δ_{RT} (min)	rRT_1 (min)	Δ_{RT} (min)	rRT_1 (min)	Δ_{RT} (min)	rRT_1 (min)	Δ_{RT} (min)
1	 Benzoin	1.68	0.10	3.44	0.59	1.97	0.35	1.49	0.00	1.10	0.25
2	 Benzoin methyl ether	0.61	0.00	1.24	0.14	0.95	0.48	1.45	0.00	0.49	0.33
3	 Hydrobenzoin	3.66	0.09	3.86	0.39	3.26	0.15	2.40	0.12	2.37	0.16
4	 Trans-stilbene oxide	0.43	0.00	1.73	1.94	1.23	0.54	1.38	0.67	0.44	0.20
5	 1-Phenylethane-1,2-diol	3.13	0.07	2.63	0.24	1.52	0.00	1.04	0.00	1.03	0.00
6	 Methyl mandelate	0.86	0.00	0.88	0.12	0.54	0.36	0.61	0.00	0.41	0.06
7	 Mephenesin	3.26	0.00	3.01	0.31	2.57	0.19	1.18	0.00	0.98	0.00
8	 1,1′-Bi-2-naphthol	6.71	0.50	5.27	0.21	4.43	0.22	4.54	0.27	4.12	0.45

Table 1 (*Continued*)

No.	Compound	PCAP		AD-H		OD-H		OJ-H		AS-H	
		Neutral compounds (Non-nitrogen containing)									
		rRT_1 (min)	Δ_{RT} (min)	rRT_1 (min)	Δ_{RT} (min)	rRT_1 (min)	Δ_{RT} (min)	rRT_1 (min)	Δ_{RT} (min)	rRT_1 (min)	Δ_{RT} (min)
9	 1,1′-Bi-2-naphthyl ditosylate	4.20	0.00	4.98	0.15	5.23	0.14	4.13	0.55	4.35	0.74
10	 2,3-*O*-Benzylidene-threitol	3.54	0.08	3.38	0.00	2.57	0.39	2.84	0.54	1.40	0.00
11	 2,2,2-Trifluoro-1-(9-anthryl) ethanol	4.20	0.10	3.54	0.00	3.70	1.43	3.67	0.82	2.49	0.72
12	 1,4-Bis(diphenylphosphino)-1,4-dideoxy-2,3-*O*-isopropylidene-threitol	2.88	0.00	5.30	0.28	3.38	0.22	0.76	0.00	2.98	0.00
13	 Benzyl mandelate	1.82	0.15	2.53	1.65	1.74	1.19	2.29	0.30	1.15	0.28
14	 γ-Phenyl-γ-butyrolactone	0.74	0.00	1.45	0.41	1.19	0.00	0.90	0.05	0.78	0.08

flow rate of 70 mL/min. For the prep-P-CAP$_{10\mu m}$ study, 30% MeOH was used, while 18% MeOH was used for prep-AS$_{10\mu m}$ to achieve similar retention. Column oven and nozzle temperature were 40 and 60 °C, respectively. Outlet pressure was 100 bar. Total volume injected was kept constant while the amount of material increased. The amount of sample injected ranged from 4.2 to 83.8 mg of material in a total volume of 1 mL of methanol.

2.7. Preparative analysis conditions for mixture A

Mobile phase consisted of 60% liquid CO_2 and 40% MeOH. The method was isocratic with a flow rate of 60 mL/min. Column oven and nozzle temperature were 40 and 60 °C, respectively. The outlet pressure was 100 bar.

2.8. Preparative analysis conditions for mixture B

The initial purification of mixture B required a mobile phase that consisted of 60% liquid CO_2 and 40% MeOH, using the prep-AD-H column. A second purification, utilizing prep-P-CAP, of mixture B used a mobile phase comprised of 65% liquid CO_2 and 35% methanol. Both of the methods were isocratic with a flow rate of 60 mL/min. Column oven and nozzle temperature were 40 and 60 °C, respectively. The outlet pressure was 100 bar.

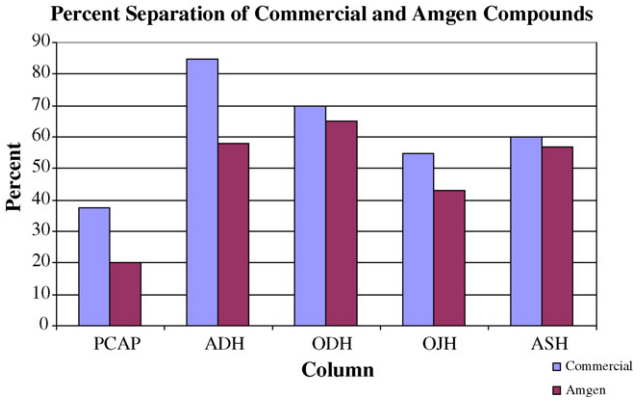


Fig. 1. The above graph shows the percentage of commercial (out of 40) and Amgen (out of 100) compounds separated by each column (P-CAP, AD-H, OD-H, OJ-H, and AS-H).

3. Results and discussion

3.1. Analytical investigation

All compounds were subjected to the same 12-min gradient method (using methanol (with or without DEA) as the modifier) via the P-CAP and the four typical derivatized polysaccharide-based

Table 2
Acidic compound structures and the corresponding relative retention time (rRT_1) of peak 1 and delta retention times (Δ_{RT}) for P-CAP, AD-H, OD-H, OJ-H, and AS-H columns

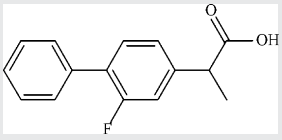
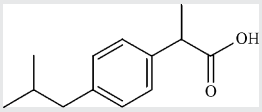
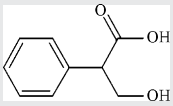
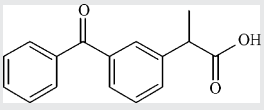
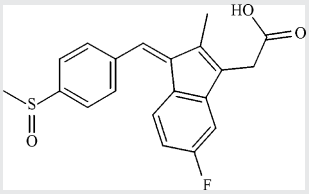
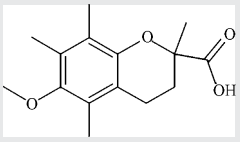
No.	Compound	PCAP		AD-H		OD-H		OJ-H		AS-H	
		Acidic compounds									
		rRT_1 (min)	Δ_{RT} (min)	rRT_1 (min)	Δ_{RT} (min)	rRT_1 (min)	Δ_{RT} (min)	rRT_1 (min)	Δ_{RT} (min)	rRT_1 (min)	Δ_{RT} (min)
15	 Flurbiprofen	3.99	0.00	2.09	1.02	2.14	0.00	2.33	0.00	1.04	0.07
16	 Ibuprofen	2.06	0.00	0.70	0.15	0.67	0.00	0.58	0.09	0.38	0.00
17	 Tropic acid	5.88	0.00	2.95	0.30	2.61	0.00	1.55	0.25	1.31	0.00
18	 Ketoprofen	4.07	0.00	2.68	0.11	2.38	0.00	1.73	0.10	1.69	0.00
19	 Sulindac	5.87	0.00	5.02	1.76	4.93	0.00	3.65	0.00	5.43	0.00
20	 Trolox-methyl ether	3.77	0.00	0.88	0.00	1.71	0.15	0.58	0.00	0.70	0.33

Table 3
Basic and neutral (nitrogen containing) compound structures and the corresponding relative retention time (rRT_1) of peak 1 and delta retention times (Δ_{RT}) for P-CAP, AD-H, OD-H, OJ-H, and AS-H columns

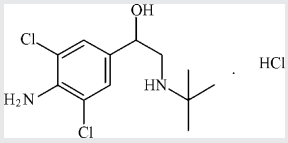
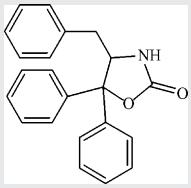
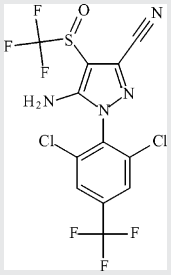
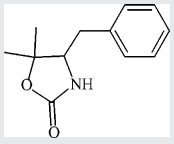
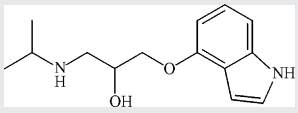
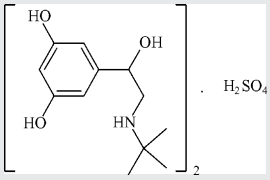
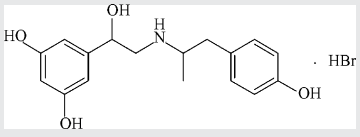
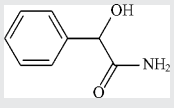
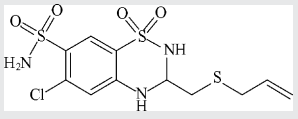
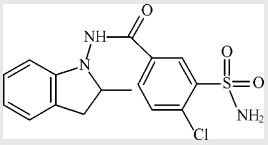
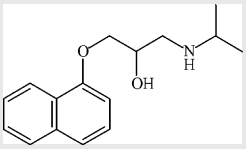
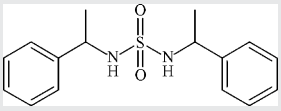
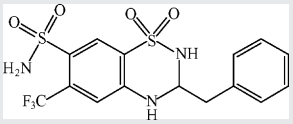
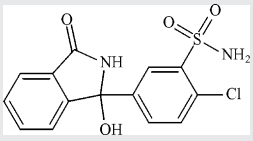
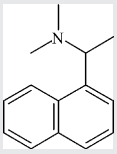
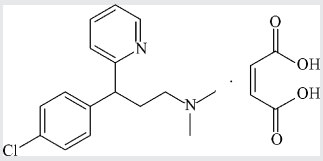
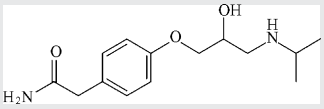
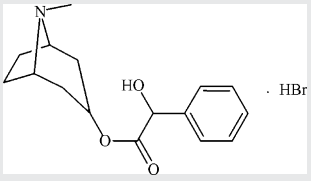
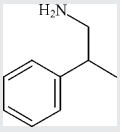
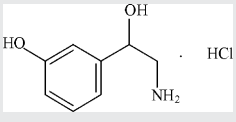
No.	Compound	PCAP		AD-H		OD-H		OJ-H		AS-H	
		Basic and neutral compounds (nitrogen containing)									
		rRT_1 (min)	Δ_{RT} (min)	rRT_1 (min)	Δ_{RT} (min)	rRT_1 (min)	Δ_{RT} (min)	rRT_1 (min)	Δ_{RT} (min)	rRT_1 (min)	Δ_{RT} (min)
21	 Clenbuterol HCl	4.59	0.00	2.92	0.12	2.49	0.17	0.69	0.06	2.05	0.42
22	 5,5-Diphenyl-4-benzyl-2-oxazolidinone	3.76	0.12	4.28	0.56	3.31	1.14	4.16	0.00	6.67	1.24
23	 Fipronil	2.27	0.35	0.25	0.00	0.67	0.07	0.36	0.00	0.28	0.00
24	 4-Benzyl-5,5-dimethyl-2-oxazolidinone	1.78	0.00	2.55	0.49	1.76	0.59	0.87	0.00	3.92	0.24
25	 Pindolol	5.44	0.00	3.57	0.18	4.38	1.53	2.55	1.13	3.20	0.19
26	 Terbutaline hemisulfate salt	6.15	0.00	2.94	0.29	3.06	0.10	0.89	0.00	2.53	0.42
27	 Fenoterol HBr	7.75	0.00	4.09	1.27	4.29	0.00	3.52	0.14	3.87	0.23
28	 Mandelamide	4.22	0.17	3.15	0.25	2.65	0.19	1.63	0.20	2.95	0.45
29	 Althiazide	8.73	0.00	7.13	0.00	4.66	0.35	4.88	0.24	5.20	0.08

Table 3 (*Continued*)

No.	Compound	PCAP		AD-H		OD-H		OJ-H		AS-H	
		Basic and neutral compounds (nitrogen containing)									
		rRT_1 (min)	Δ_{RT} (min)	rRT_1 (min)	Δ_{RT} (min)	rRT_1 (min)	Δ_{RT} (min)	rRT_1 (min)	Δ_{RT} (min)	rRT_1 (min)	Δ_{RT} (min)
30	 Indapamide	6.57	0.06	5.07	0.16	5.47	0.28	5.22	0.06	5.05	0.36
31	 Propranolol HCl	4.18	0.00	3.20	0.60	4.07	0.72	1.57	0.25	2.36	0.00
32	 *N*,*N*′-Bis(α-methylbenzyl)sulfamide	4.37	0.08	3.89	1.26	2.70	0.19	2.04	0.54	3.66	0.00
33	 Bendroflumethiazide	8.45	0.15	4.36	0.17	4.49	0.07	4.39	0.28	4.67	0.16
34	 Chlorthalidone	7.99	0.27	6.28	0.37	4.61	0.23	4.52	0.14	6.40	0.13
35	 *N*,*N*′-Dimethyl-1-(1-naphthyl)ethylamine	1.71	0.00	2.08	0.28	1.68	0.00	0.56	0.00	0.77	1.71
36	 Chlorpheniramine maleate	0.71	0.00	0.69	0.15	1.00	0.00	0.59	0.08	0.39	0.00
37	 Atenolol	5.22	0.00	4.34	0.00	4.02	0.72	1.71	0.00	4.75	0.00
38	 Homatropine HBr	8.19	0.08	3.67	0.16	3.40	0.75	1.02	0.11	2.79	0.10
39	 β-Methyl-phenethylamine	3.30	0.00	1.61	0.44	1.33	0.27	0.50	0.00	0.98	0.00
40	 Norphenylephrine HCl	6.42	0.00	4.16	0.32	3.34	0.00	2.52	0.00	3.17	0.00

CSPs (AD-H, OD-H, OJ-H, and AS-H) that were investigated. Gradient elution with SFC has been previously shown to be very successful in other labs and has been implemented in their screening process as well [22,23]. The method is simple and quite useful for determining the proper column for a chiral separation in a rapid and efficient manner.

Tables 1–3 show the commercial compounds used in the study along with the results (rRT values of the first peak (rRT_1) as well as the Δ_{RT} values) for the P-CAP and the amylose- and cellulose-based CSPs. Table 1 displays the rRT_1 and Δ_{RT} values for neutral, non-nitrogen containing compounds. The P-CAP and OJ-H columns separated 50% and 57% of these compounds, respectively. Both columns failed to separate four compounds in common. Though no single column provided separation for all compounds, AD-H and OD-H both separated 86% of the compounds which was significantly more than the other CSPs.

Looking at Table 2, it is clear that P-CAP did not provide separation for the acidic commercial compounds that were analyzed, while the derivatized polysaccharide-based CSPs did produce separation for several of those compounds. It is not expected that P-CAP has or will have difficulty in separating all acidic compounds in general [10]. At least one of the Amgen compounds that separated by P-CAP was acidic. Further exploration of various acidic compounds and/or mobile phases and additives will need to be conducted in future studies. The most successful column for the separation of acidic compounds was the AD-H column with a success rate of 83%.

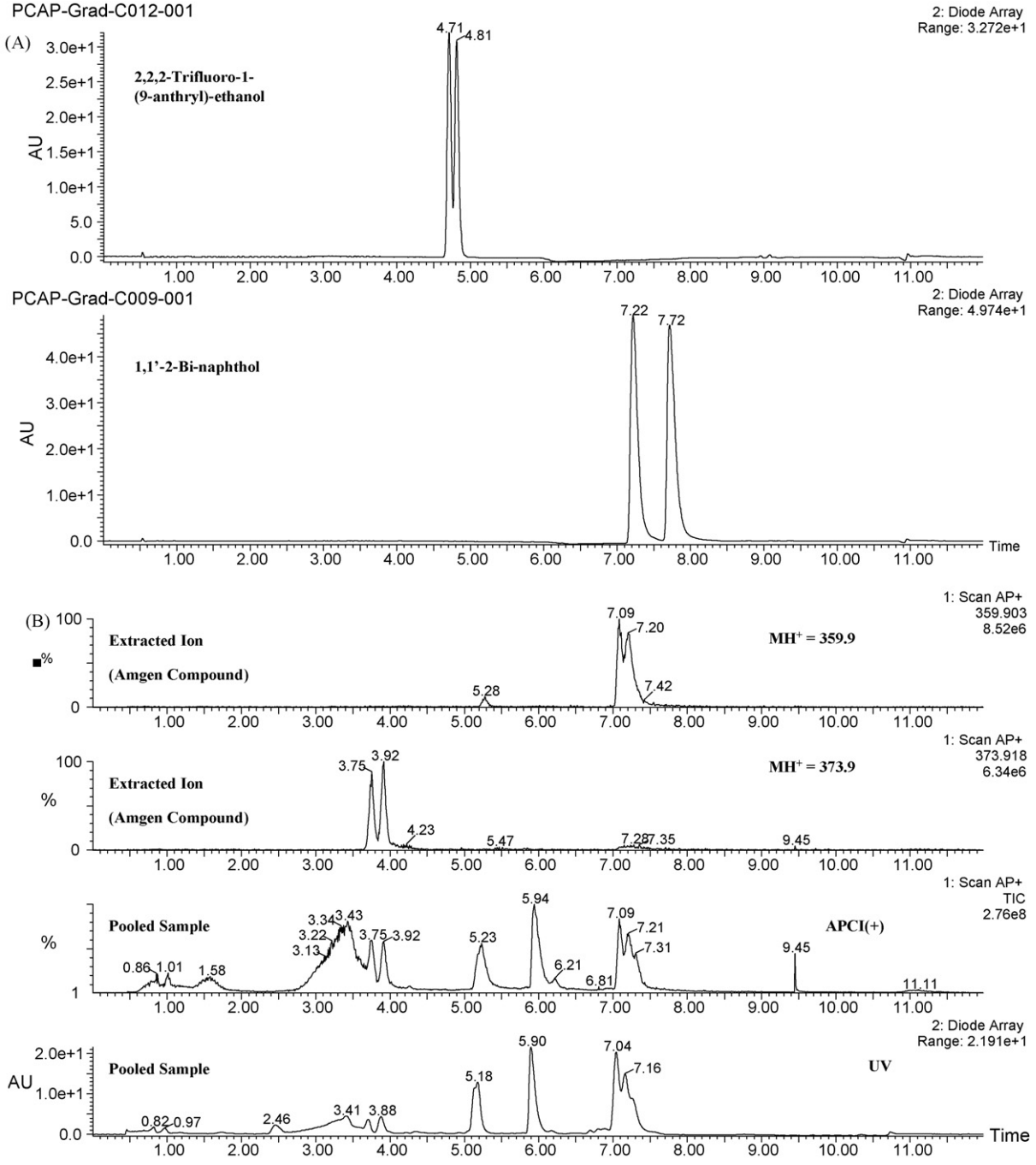


Fig. 2. Analytical SFC chromatograms showing the separation of selected commercial (A) and Amgen (B) samples using the (*R*,*R*) P-CAP column. All analyses were performed using the same standard, aforementioned 12 min gradient method and conditions.

OD-H, however, did not perform as well with acidic compounds compared to the neutral, non-nitrogen containing compounds. The OD-H column only separated 17% of the acidic compounds. Separation was noted in 33% and 50% of the compounds via the AS-H and OJ-H columns, respectively.

Table 3 shows the rRT_1 and Δ_{RT} values for basic and neutral, nitrogen containing compounds. Again, the P-CAP column showed separation for the least number of these compounds. P-CAP separated 40% of the compounds, while the AD-H and OD-H columns separated the most compounds (85% and 80%, respectively). The percentage of compounds separated by OJ-H and AS-H were similar (60% and 65%, respectively) but less than the AD-H and OD-H columns.

The results of the percentage of successful separations observed in the investigation are displayed in Fig. 1. This figure shows a similar trend in the percentage of successful separations. P-CAP separated the least number of compounds for both the Amgen and commercial compounds. The AD-H and OD-H columns, however, reversed their effectiveness when comparing the Amgen and commercial compounds. Example chromatograms, including Amgen compounds together with commercial compounds, are shown in Fig. 2.

Of the total 140 compounds that were analyzed, P-CAP demonstrated separation of 25% of the compounds, while each of the derivatized polysaccharide-based CSPs separated at least 46%. Eight (compound numbers 3, 8, 13, 28, 30, 33, 34, and 38) of the 40 commercial compounds that were investigated showed separation by every column. Each compound that separated with P-CAP also separated with at least one of the four other columns. No separations were observed that were unique to P-CAP. Two compounds demonstrated Δ_{RT} values with P-CAP that were greater than the other columns showing successful separation. Fipronil had a greater Δ_{RT} (0.35) with the P-CAP column compared to the only other successful column (OD-H) with a value of 0.07, while the Δ_{RT} for 1,1′-bi-2-naphthol with P-CAP was 0.50 compared to 0.21, 0.22, 0.27 and 0.45 for AD-H, OD-H, OJ-H and AS-H, respectively. The AD-H and OD-H columns demonstrated separation for the largest percentage of total compounds. Considering the AD-H and OD-H columns, all commercial compounds separated with P-CAP were separated by either AD-H or OD-H. Two Amgen compounds, which were separated with P-CAP, could not be separable with either AD-H or OD-H. The OJ-H column separated the least number of compounds among all derivatized polysaccharide-based CSPs.

To see how the average rRT_1 and Δ_{RT} values of the commercial compounds compared among the columns, rRT_1 and Δ_{RT} values were averaged for each of the five columns. Fig. 3 graphically illustrates the results of the calculations. The derivatized polysaccharide-based CSPs follow a trend that the increase in average rRT_1 pairs with an increase in average Δ_{RT}. For example, the OJ-H column had an average rRT_1 value of 2.08 min and an average Δ_{RT} value of 0.17 min, while the average rRT_1 and Δ_{RT} values (2.51 and 0.24 min, respectively) of the AS-H column were greater. Though the averaged data follows this trend, a quick look at the individual data points (see Tables 1–3) shows that the results do not always follow the pattern. For example, the rRT_1 and Δ_{RT} values of benzoin methyl ether on AD-H are 1.24 and 0.14 min, respectively. The Δ_{RT} (0.33 min) on the AS-H column, however, is greater than that of the AD-H column, even though the rRT_1 (0.49 min) is less than half of that of AD-H. Unlike the trend observed for the derivatized polysaccharide-based CSPs, a greater average rRT_1 does not always indicate an increased average Δ_{RT} value. The average rRT_1 of the P-CAP column was 4.12 min, which was greater than any of the derivatized polysaccharide-based CSPs; however, P-CAP demonstrated the least average Δ_{RT} of 0.06 min, which is less than half of that observed for the other four columns, suggesting that increased retention time does not always indicate better chiral separation. Observed chiral separation is the result of the difference in retention between the two enantiomers [24]. Overall, it appears that P-CAP has much less chiral discrimination power in spite of longer retention time than when compared to the derivatized polysaccharide-based CSPs for the compounds analyzed in this study.

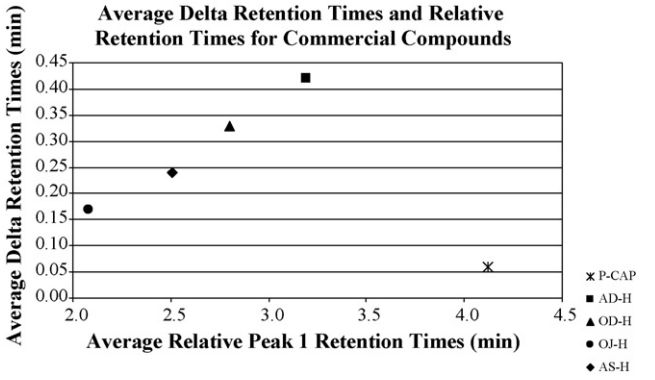


Fig. 3. Comparison of the average delta retention times to the average relative retention times of commercial compounds using the P-CAP, AD-H, OD-H, OJ-H, and AS-H columns.

Interestingly enough, the trend (AD-H > OD-H > AS-H > OJ-H) demonstrated by the amylose and cellulose columns for the chiral discrimination of commercial compounds, with regard to average rRT_1 and Δ_{RT} values, was followed by the percentage of successful separations found in Fig. 1. As the percentage of successful separations increased, the average rRT_1 and Δ_{RT} values increased in the same order.

3.2. Loading study

Part of understanding the usefulness of a column for chiral separation is to investigate its ability to load sample. The prep-P-$CAP_{10\mu m}$ and prep-$AS_{10\mu m}$ columns were both studied using 1,1′-bi-2-naphthol. 1,1′-bi-2-naphthol was chosen due to the similar Δ_{RT} values of 0.50 and 0.45 min for the analytical P-CAP and AS-H columns, respectively. Percentage of MeOH used for each column was chosen such that the α values were similar. The α values for the prep-P-$CAP_{10\mu m}$ and prep-$AS_{10\mu m}$ columns were 1.24 and 1.31, respectively. These values were considered similar enough for the purpose of the loading study. The loading experiment was carried out using injection amounts ranging from 4.2 to 84 mg for both columns. Resolution of the prep-$AS_{10\mu m}$ column remained consistently better than prep-P-$CAP_{10\mu m}$, thus demonstrating higher loadability with prep-$AS_{10\mu m}$ over the range of sample amount investigated. The resolutions of the prep-$AS_{10\mu m}$ changed from 3.05 to 1.15, while those of prep-P-$CAP_{10\mu m}$ changed from 1.83 to 0.90. However, there was no dramatic change in resolution trends between the two columns at any of the data points.

3.3. Application of P-CAP

P-CAP showed a clear advantage over the other four columns for the separation of a complex mixture (mixture A) comprised of enantiomers and a major achiral impurity in addition to several minor impurities (see Fig. 4). This mixture contained three major peaks, but only two were predicted to be enantiomeric. With P-CAP, all three were well separated, analytically, in less than

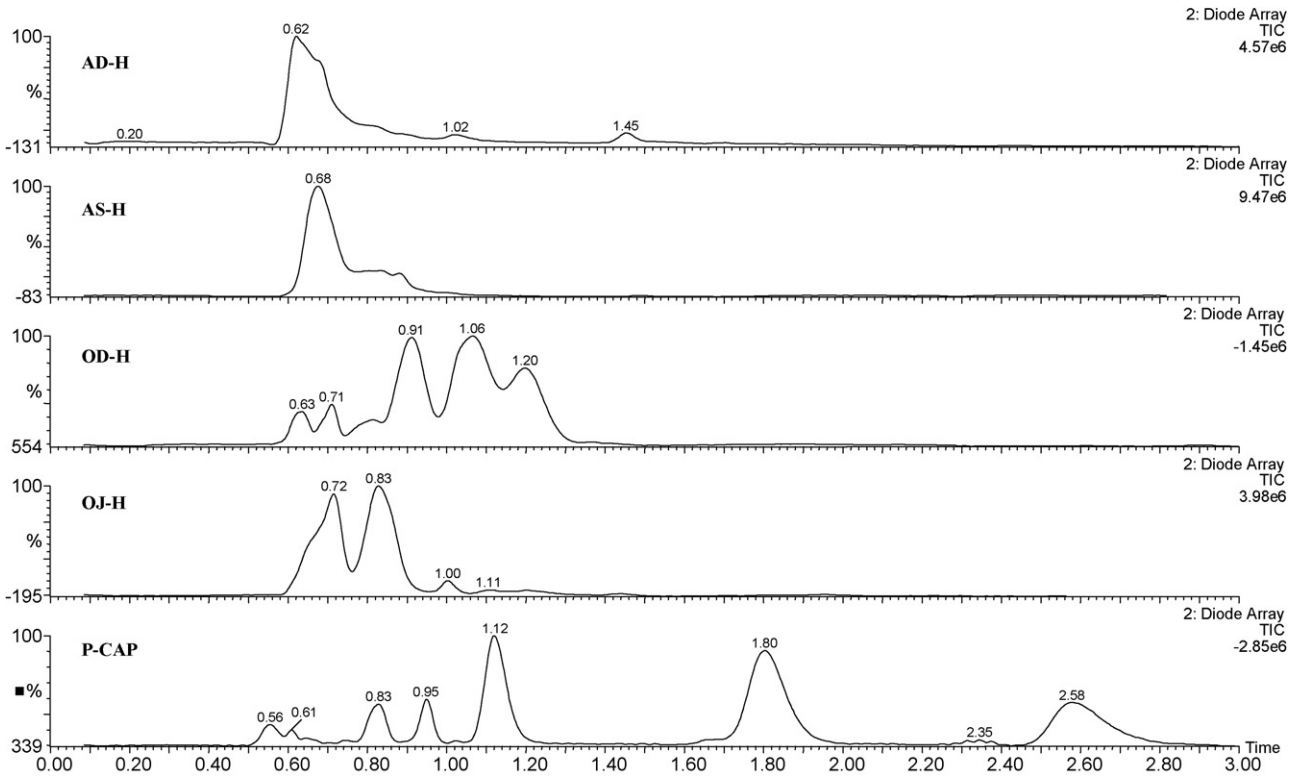


Fig. 4. Analytical chromatograms of mixture A with AD-H, AS-H, OD-H, OJ-H, and (*R*,*R*) P-CAP columns. The mobile phase consisted of 60% liquid CO_2 and 40% methanol at a flow rate of 4.0 mL/min. Oven temperature and outlet pressure were 35 °C and 100 bar, respectively.

3 min. By exploring different organic modifiers and percentages, the derivatized polysaccharide columns may have adequate separation; however, P-CAP allows the ability to "invert" elution order of enantiomers "at will," thus making it a good choice for the purification.

Mixture A was purified by preparative SFC (see Fig. 5) utilizing the *R*,*R* form of prep-P-CAP. The three fractions collected from the purification were analyzed via *R*,*R* (see Fig. 6A) and *S*,*S* (see Fig. 6B) forms of P-CAP to identify the enantiomeric pair. It is reasonable to assume that the peaks resulting from the enantiomers should reverse in their elution order while not affecting the retention time of the major achiral impurity. By changing the elution order of peaks 1 and 3 (using (*S*,*S*) P-CAP in place of (*R*,*R*) P-CAP), it was obvious which peaks were enantiomers (peaks 1 and 3) and which of the three peaks (peak 2) was a major impurity. While the elution order changed for peaks 1 and 3 with the use of the different forms of the P-CAP column, the retention time of peak 2 remained constant.

Other studies have shown that elution order can be changed for the separation of some chiral compounds using the AD-H column by varying organic modifiers, thereby altering the chiral cavities [25,26]. However, this is not as reliable or predictable and is more

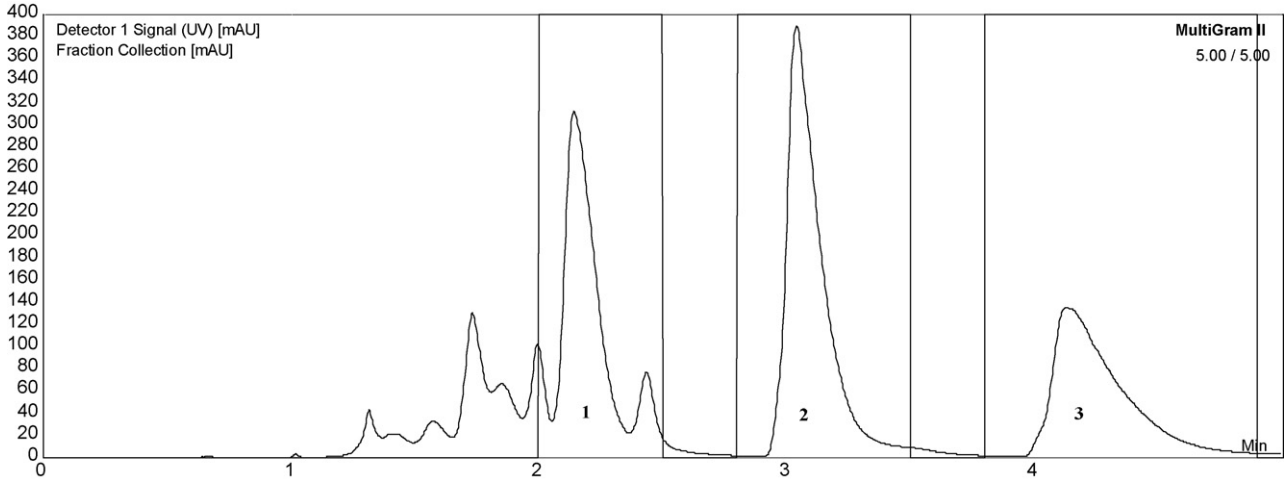


Fig. 5. Preparative chromatogram of 5 mg of mixture A utilizing prep-P-CAP. The mobile phase consisted of 50% liquid CO_2 and 50% methanol. The oven temperature was 40 °C, and the outlet pressure was 100 bar. Total flow was 60 mL/min.

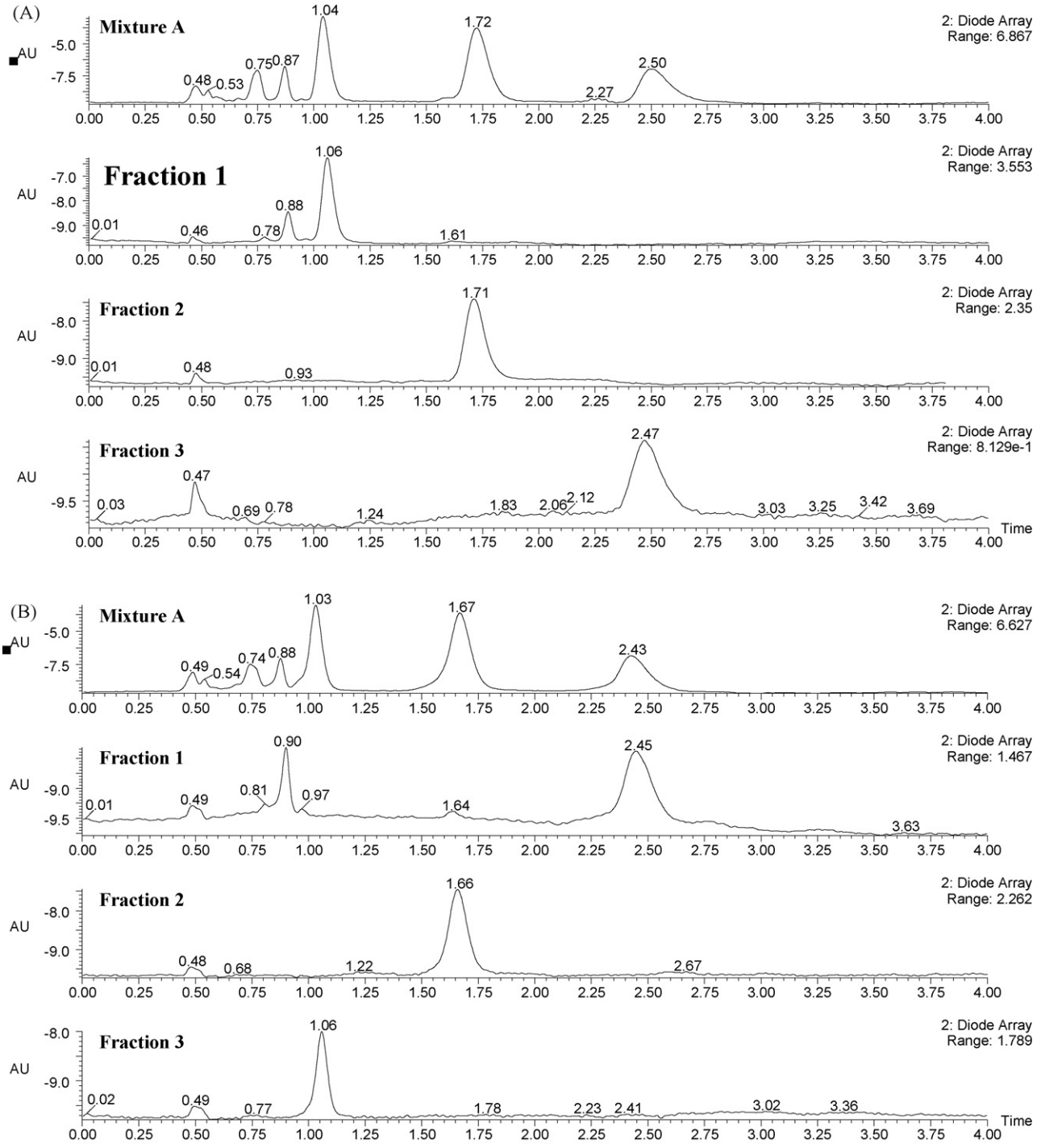


Fig. 6. Analytical chromatograms of mixture A with the (*R*,*R*) P-CAP (A) and (*S*,*S*) P-CAP (B) columns. Mobile phase consisted of 60% liquid CO_2 and 40% methanol at a flow rate of 4.0 mL/min. Oven temperature and outlet pressure were 35 °C and 100 bar, respectively. Fractions were obtained from preparative chromatography using (*R*,*R*) P-CAP and numbered according to the elution order using that column.

time-consuming than simply switching between the two different forms of the P-CAP columns.

Another successful application of the prep-P-CAP column was in the removal of an achiral impurity from a mixture (mixture B) that consisted of several achiral impurities, in addition to a small amount of an opposite enantiomer. The initial purification (see Fig. 7A) was required to remove the opposite enantiomer. After purification, purity analysis using reversed phase chromatography revealed that all but one of the achiral impurities was removed, thus requiring additional purification. The prep-P-CAP column was then found to provide a very different selectivity (compared to prep-AD-H) for the impurity and allowed a very nice separation (see Fig. 7B) under similar conditions. Though an achiral column may have been just as viable for this final purification step, it was the P-CAP column that was first explored and found to demonstrate the desired separation.

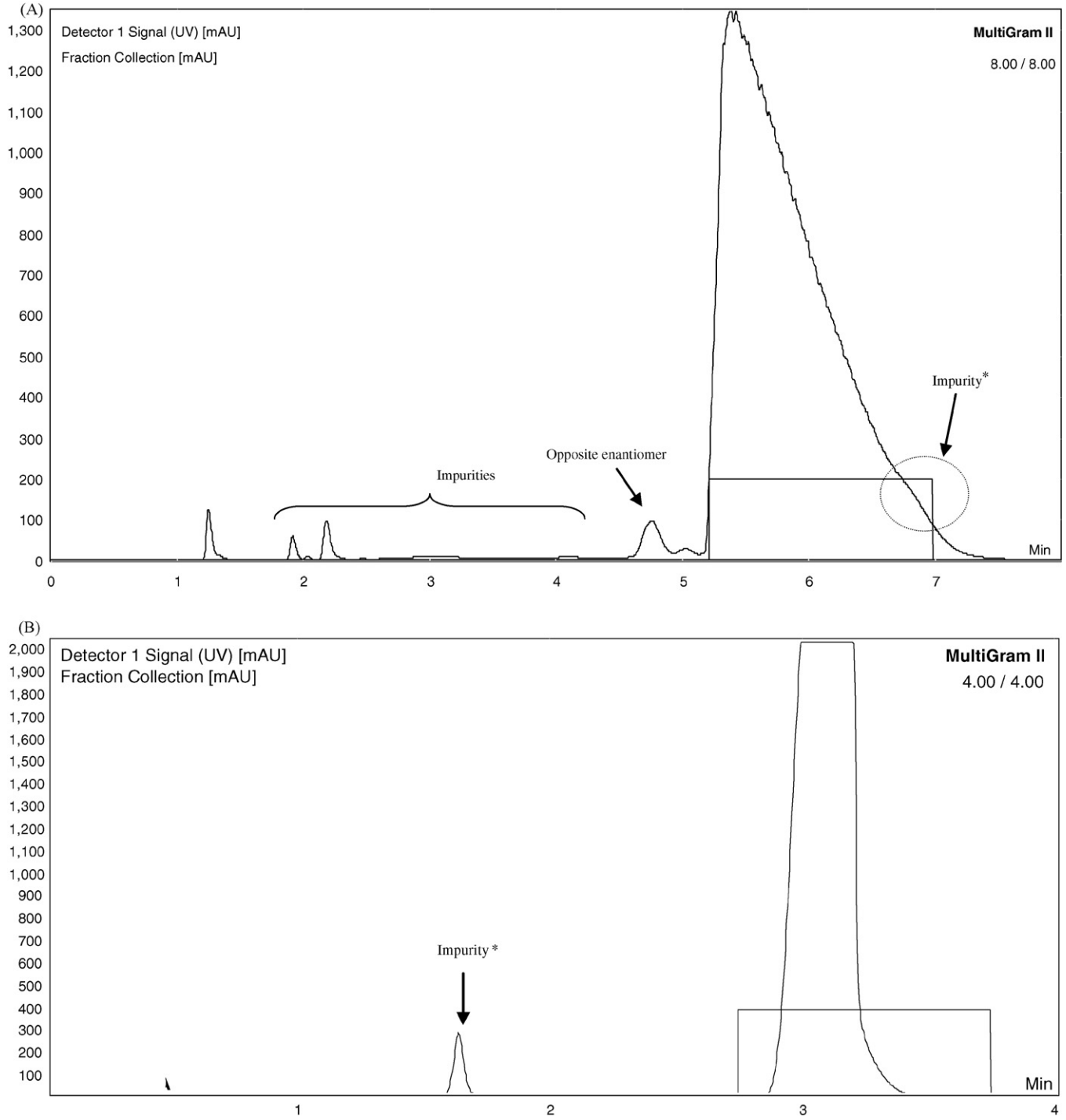


Fig. 7. Preparative chromatograms showing the purification of a sample mixture (mixture B) containing achiral impurities in addition to an undesired enantiomer. The initial purification (A) utilized a prep-AD-H column with a mobile phase that consisted of 60% liquid CO_2 and 40% methanol. A second purification (B) using the prep-P-CAP was required to remove an achiral impurity. This purification used a mobile phase that consisted of 65% liquid CO_2 and 35% methanol.

4. Conclusion

P-CAP did show that it can successfully provide separations of various compounds by SFC. According to the current results, it does not offer an overall advantage over the derivatized polysaccharide-based CSPs when considering separation success percentages resulting from the gradient screening method. P-CAP can be a powerful tool for manipulating elution order which can be critical in the determination of enantiomer peak pairs vs. achiral impurity. The success of P-CAP in the separation of a complex mixture of enantiomers and a major impurity was extremely helpful, and it was also helpful in the separation of an achiral impurity from a mixture containing the achiral impurity and desired compound.

Overall, the P-CAP column will continue to be a part of the screening process for method development when the four derivatized polysaccharide-based CSPs fail to show separation. More varied mobile phases and additives may be needed to better investigate the potential of this column.

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