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Effect of amine mobile phase additives on chiral subcritical fluid chromatography using polysaccharide stationary phases

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Abstract

Increased retention and selectivity in the subcritical fluid chromatography (SFC) of various amine compounds on polysaccharide chiral stationary phases (CSP) was observed upon incorporation of cyclic amines into the modifier. The retention increases are most pronounced with 2-propanol and are almost absent when methanol is used as modifier. This suggests that the effect may arise from a restriction to the modifier access to the binding site required to effect elution. The effect of the amine additives in SFC does not remain after their removal from the mobile phase. Findings were applied to the development of a 5 min separation of amphetamine and methamphetamine enantiomers.
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Keywords: Mobile phase composition; Chiral stationary phases, SFC; Amines

1. Introduction

Recent work [1–4] has shown that acidic and basic mobile phase additives used to improve peak shapes in chiral HPLC may also affect enantioselectivity. The separations of acidic phenylalanine analogs on a CHIRALPAK® AD® column were affected by both the pK_a's and hydrophobicity of acidic additives [1]. This was interpreted as being indicative of both ion suppression and ion pair formation effects. Separations of phenylalanine analogs with both free amine and acid functionalities [2] were altered by the inclusion of amine additives. General observations included that primary amines were more effective additives than more commonly used secondary and tertiary amines. In many cases, additives gave slight increases in selectivity through a larger decrease in retention of the first eluting enantiomer than of the second. Decreased retention is viewed as arising from competition for binding opportunities between the amine additive and the analytes. More interesting were the observations of increased retention in response to inclusion of amine additives. These cases often gave dramatic increases in selectivity. The size and shape of the additive strongly influenced the retention increase, leading to the suggestion that the amine was preventing access of modifier seeking to displace tightly bound enantiomer. Unfortunately, interpretation of these amine effects was somewhat clouded by the requirement for acidic additives in the mobile phase to elute acidic analytes from polysaccharide chiral stationary phases (CSP).

Acidic additives are not required for such applications when subcritical fluid chromatography (SFC) is used. This is attributed to the "acidic" nature of carbon dioxide. It is worth noting that a protic modifier is also required. Amine additives have been used in SFC occasionally with the intent of improving peak shape [5–8] of amine analytes. The common interpretation is that amine additives mask silanols that contribute to non-specific retention of such amines. Diminishing non-specific interactions would decrease retention but should also increase observed selectivity. Amine additives would also be expected to compete with amine analytes for specific binding sites giving decreased retention but mixed effects on selectivity. This is the typical observation for a broad range of amine analytes [8]. Admittedly, amine additives have not been examined in depth in SFC. This may be due to the relative lack of success of the technique with amine analytes. Amines often fail to elute, or give peaks so distorted that optimization is not attempted.

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Any explanation of additive effects in SFC must also consider the "acidic" nature of carbon dioxide. Incorporation of an amine additive should alter the acidity of the mobile phase. It was found that acidic analytes no longer elute in SFC conditions when amine additives are used. This implies that the carbon dioxide is no longer "acidic" enough for these analytes to elute. It is possible that distorted amine peaks arise in SFC from a protonation–deprotonation equilibrium induced by the acidic nature of the mobile phase. Addition of an amine additive could force deprotonation and improved peak shape would result from simplification of the equilibrium. It is unlikely that the effects of amine additives can be interpreted this simply. Primary, secondary and tertiary amines would be expected to have different effects on this equilibrium. This is rarely observed to be true [8]. Deprotonated amine analytes should be less polar and retention should decrease.

Another complicating factor is the possibility of carbon dioxide forming transient complexes with amine groups [6,9–12]. This factor has been proffered as an explanation for different selectivity for amine analytes between SFC and HPLC. Spectroscopic evidence [9,11] is compelling. Such an interaction must be expanded to include amine groups present on the stationary phase. Amine additives

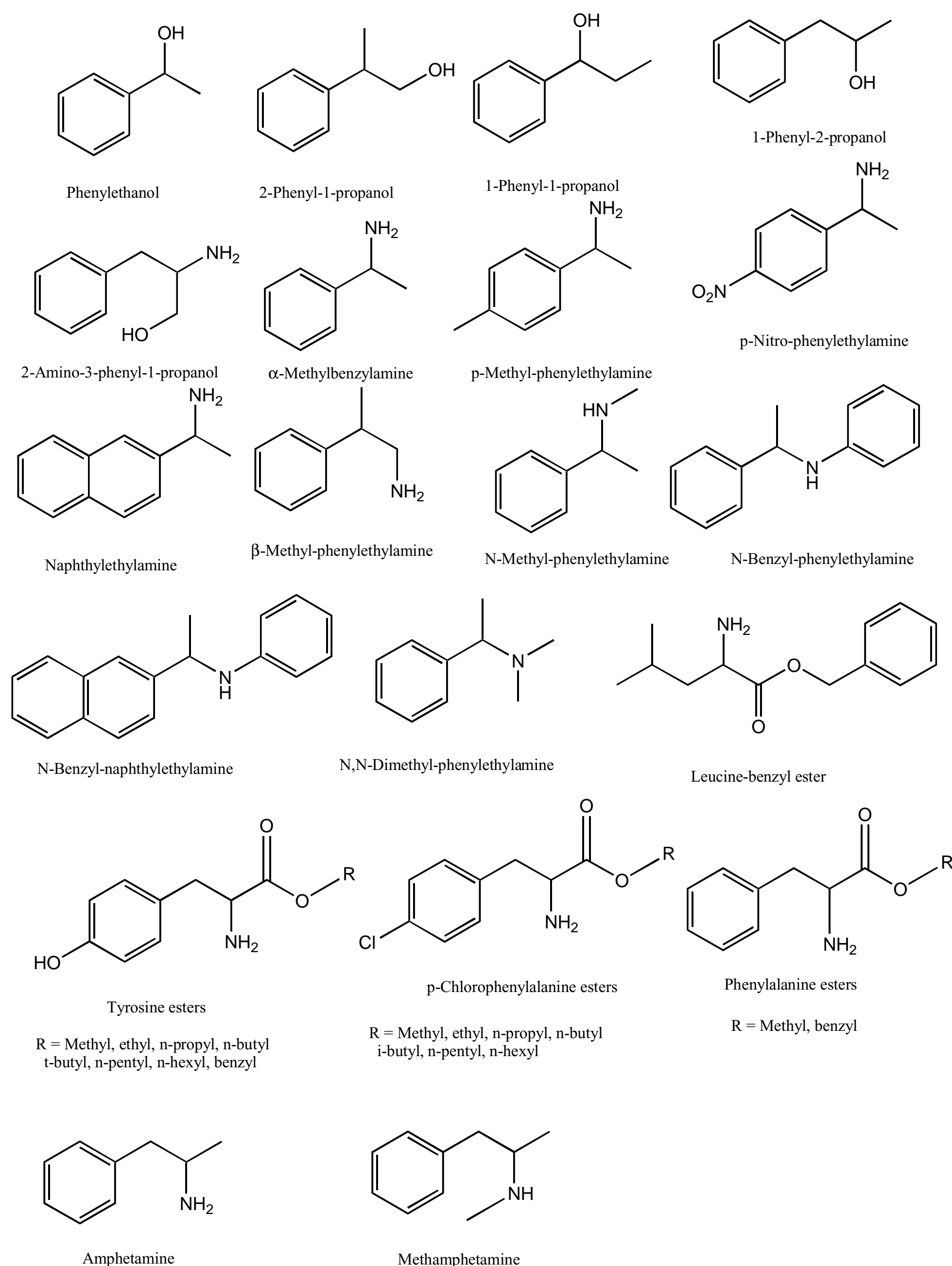

Fig. 1. Structure of probes used in this study.

would also complex with carbon dioxide. It is unclear what impact amine additives would have on these interactions.

Any attempt to clarify the use of amine additives in SFC should involve a broad screening approach. To this end, 32 different compounds were screened against nine different primary amine additives. The compounds (Fig. 1) included 4 aromatic alcohols, 1 amine alcohol, 5 aromatic primary amines, 3 secondary amines, 1 tertiary amine and 18 amino acid esters. The amine additives included straight chain aliphatic amines from *n*-propyl to *n*-hexyl and cyclic amines from cyclopropyl to cyclooctylamine. Based on obtained screening results, additional experimentation was performed on a fewer number of samples and findings were applied to the development of a practical separation.

Table 1
Effect of amine additives on the retention of α-methylbenzylamine

Additive	k'_1	k'_2	α	Rs[a]
None	2.62	2.81	1.07	0.8
n-Propylamine	2.18	2.18	1.00	0
n-Butylamine	2.15	2.15	1.00	0
Amylamine	2.11	2.22	1.05	0.6
n-Hexylamine	2.16	2.26	1.04	0.5
Cyclopropylamine	2.24	2.43	1.09	1.0
Cyclobutylamine	2.58	2.58	1.00	0
Cyclopentylamine	3.00	4.02	1.34	3.6
Cyclohexylamine	2.67	3.29	1.23	2.5
Cycloheptylamine	3.49	4.32	1.24	2.8
Cyclooctylamine	3.89	4.79	1.23	2.8

[a] Rs: estimated resolution.

2. Experimental

2.1. Reagents

All reagents used in this study were reagent grade or better. Probe molecules and amine additives were obtained from either BACHEM Biosciences (Philadelphia, PA) or Sigma–Aldrich (St. Louis, MO). Ethanol was obtained from J.T. Baker (Phillipsburg, NJ) and methanol and 2-propanol were from Pharmco (Brookfield, CT).

2.2. Chromatography

Chromatographic studies were performed on Berger Supercritical Fluid Chromatographs (Berger, Newark, DE) equipped with autosampler, thermostated-column device and a variable-wavelength UV detector. CHIRALPAK® AD® and AD-H® columns (250 mm × 4.6 mm) were received from Chiral Technologies (Exton, PA). Chromatographic screening studies were performed at 40 °C with a 1.5 ml/min flow rate, 200 bar back pressure, 10% ethanol modifier with or without 2% (v/v) amine additive. Alternative conditions are described in the text.

3. Results and discussion

A large amount of data was generated in screening experiments which will not be presented in detail. None of the amine additives had a significant effect on the chromatography of the alcohol probes. The effects on the primary amines were more interesting. Table 1 shows that straight chain aliphatic amine additives decrease retention of both enantiomers of α-methylbenzyl amine, consistent with a competition mechanism for binding sites between analyte and additive. Cyclic amines larger than cyclobutyl however gave increased retention times and enhanced selectivity. The primary amine additives have very similar pK_b values. The differences in their chromatographic effects must arise from attributes other than their effects on analyte ionization. Similar primary amines (*p*-methylphenylethylamine, *p*-nitrophenylethylamine, naphthylethylamine, β-methylphenylethylamine) gave similar results although the selectivity enhancements were not as pronounced. 2-Amino-3-phenyl-1-propanol is a primary amine that also contains an alcohol group. Selectivity was observed only with cyclobutylamine. Resolution values are estimated from chromatographic measurements.

All amine additives gave decreased retention for secondary amine probes. There were occasional observations of increased selectivity. *N*-Methyl-phenylethylamine showed a significant increase in selectivity (α: 1.00 → 1.47) with cycloheptylamine. Cyclopropylamine gave an increased selectivity (α: 1.00 → 1.41) for *N*-benzyl-naphthylethylamine. The tertiary amine probe (*N*,*N*-dimethyl-phenylethylamine) showed decreased retention with all amine additives but only a slight increase in selectivity (α: 1.00 → 1.11) with cyclopropylamine.

The amino acid esters gave the most interesting results in the screening experiments. Straight chain aliphatic amines had relatively little effect on the chromatography of these probes while cyclic amines gave increased retention and often dramatically increased selectivity. Results for phenylalanine-methyl ester are shown in Table 2. Straight chain amine additives give slight increases in retention. Cyclic amines give significant retention increases. Retention times increase with ring size from cyclopropyl to cyclooctylamine. Selectivity tends to increase with retention although eventually the retention increase of the first eluting enantiomer matches that of the second. Results for tyrosine-methyl ester were similar (Table 2). This probe differs from the phenylalanine ester by the presence of the phenolic group. The magnitude of the increased retention arising from this change suggests its involvement in an additional binding interaction. Leucine-benzyl ester showed slight increases in retention with straight chain aliphatic amine additives. Cyclobutylamine and cyclopentylamine give dramatically increased retention but without selectivity. The retention increase was less dramatic with cyclohexylamine but the increase was enantio-selective.

Table 2
Effect of amine additives on the retention of tyrosine-methyl ester and phenylalanine-methyl ester

Additive	Tyrosine-methyl				Phenylalanine-methyl			
	k'_1	k'_2	α	Rs[a]	k'_1	k'_2	α	Rs[a]
None	6.73	6.73	1.00	0	1.49	1.49	1.00	0
n-Propylamine	6.48	7.18	1.11	1.5	1.63	1.72	1.05	0.5
n-Butylamine	6.21	6.96	1.12	1.7	1.62	1.76	1.09	0.9
Amylamine	6.64	7.46	1.12	1.7	1.55	1.66	1.07	0.7
n-Hexylamine	6.55	7.32	1.12	1.7	1.63	1.80	1.10	1.0
Cyclopropylamine	7.25	7.25	1.00	0	1.33	1.46	1.10	0.9
Cyclobutylamine	8.76	9.54	1.09	1.2	1.94	2.04	1.05	0.5
Cyclopentylamine	11.72	20.53	1.75	6.5	2.85	3.47	1.22	2.3
Cyclohexylamine	10.12	13.36	1.32	3.6	2.37	3.12	1.32	3.1
Cycloheptylamine	14.73	23.48	1.59	5.6	3.19	3.86	1.21	2.3
Cyclooctylamine	15.97	25.25	1.58	5.6	3.94	5.03	1.27	2.8

[a] Rs: estimated resolution.

Table 3 shows the effect of cyclopentylamine on retention and selectivity as a function of different ester groups for tyrosine and *p*-chlorophenylalanine. As the size of the tyrosine ester increases, retention without additive decreases. This is consistent with a poorer fit in the binding site. That the bulkier *t*-butyl ester shows lower retention than *n*-butyl favors this interpretation over any change in solubility in the mobile phase. Incorporation of cyclopentylamine into the modifier increased retention dramatically. With tyrosine esters selectivity was most increased for the larger esters. Although larger esters do not fit into the binding site as well, they appear more effective in conjunction with the amine additive at restricting the modifier's access to disrupt binding interactions. This trend is not as noticeable with the *p*-chlorophenylalanine esters.

Based on screening results, a closer examination of the effects of amine additives was made with five selected compounds. Three amino acid esters were chromatographed on a new CHIRALPAK® AD® column using methanol, ethanol and 2-propanol modifiers doped with cyclohexylamine at different levels. Different alcohol modifiers are known to give different enantio-selectivity in SFC when using polysaccharide stationary phases. This is commonly attributed to changes in the tertiary structure of the polymer although other factors likely play a role as well. Results are given in Tables 4 and 5. In the absence of amine additive, chromatography was quite similar for all three modifiers. Methanol gave a partial separation of tyrosine-methyl ester not observed with other modifiers. The response to added cyclohexylamine was less pronounced with methanol modifier for all three probes. Additive-induced retention increases were more dramatic for 2-propanol modifier than for ethanol. This is consistent with a restricted access mechanism of the cyclic amine effect. Retention increased with additive level. Selectivity typically increased as well. With 2-propanol modifier going from 0.4 to 1.0% cyclohexylamine, the retention of the first eluting enantiomer increased as much as the second enantiomer. Fig. 2 shows

Table 3
The effect of cyclopentylamine additive on the retention of tyrosine and *p*-chlorophenylalanine (*p*-Cl-Phe-) esters

Ester probe	No additive				Cyclopentylamine			
	k'_1	k'_2	α	Rs[a]	k'_1	k'_2	α	Rs[a]
Tyrosine-methyl	6.24	6.24	1.00	0	10.56	18.57	1.76	6.5
Tyrosine-ethyl	5.03	5.50	1.09	1.1	8.78	23.24	2.65	9.4
Tyrosine-*n*-propyl	5.03	5.67	1.13	1.5	9.08	24.75	2.73	9.6
Tyrosine-*n*-butyl	4.86	5.13	1.06	0.7	8.48	24.84	2.93	10.0
Tyrosine-*t*-butyl	3.72	4.20	1.13	1.5	5.96	13.10	2.20	8.0
Tyrosine-*n*-pentyl	4.83	4.96	1.03	0.4	8.08	23.43	2.90	9.9
Tyrosine-*n*-hexyl	4.68	4.98	1.06	0.7	8.15	24.78	3.04	10.2
p-Cl-Phe-methyl	1.90	1.90	1.00	0	3.55	4.59	1.29	3.1
p-Cl-Phe-ethyl	1.58	1.58	1.00	0	3.31	5.44	1.64	5.5
p-Cl-Phe-propyl	1.62	1.62	1.00	0	3.16	5.44	1.72	5.9
p-Cl-Phe-*n*-butyl	1.60	1.60	1.00	0	3.26	6.00	1.84	6.6
p-Cl-Phe-*i*-butyl	1.44	1.66	1.16	1.4	2.82	4.78	1.70	5.7
p-Cl-Phe-*n*-pentyl	1.64	1.64	1.00	0	3.20	5.77	1.80	6.3
p-Cl-Phe-*n*-hexyl	1.91	1.91	1.00	0	4.73	5.39	1.14	1.7

[a] Rs: estimated resolution.

Table 4
The effect of different modifiers and cyclohexylamine additive levels on the retention of amino acid esters

Modifier	Phenylalanine-methyl ester					Tyrosine-methyl ester			
	% CHA	k'_1	k'_2	α	Rs	k'_1	k'_2	α	Rs
Methanol	0	0.90	0.96	1.07	0.75	1.39	1.68	1.21	1.31
	1.0	0.96	1.06	1.10	1.04	1.48	1.69	1.14	1.39
Ethanol	0	0.88	0.88	1.00	0	1.74	1.74	1.00	0
	0.1	0.87	0.95	1.09	0.83	1.65	1.70	1.03	0
	0.4	1.10	1.35	1.22	1.84	1.99	2.49	1.25	2.56
	1.0	1.32	1.75	1.33	2.81	2.31	3.38	1.47	4.41
2-Propanol	0	0.84	0.84	1.00	0	2.31	2.31	1.00	0
	0.1	0.97	1.31	1.35	1.22	2.55	4.83	1.89	4.21
	0.4	1.34	2.54	1.89	5.21	3.51	11.54	3.28	9.86
	1.0	2.30	4.22	1.84	4.86	5.10	18.13	3.55	10.5

Rs: resolution calculated by Berger software.

the chromatograms of phenylalanine methyl ester obtained with different levels of cyclohexylamine in ethanol modifier. Resolution and selectivity increased with additive level. Fig. 3 shows chromatograms of tyrosine-methyl ester at three levels of additive in 2-propanol. Retention increases were dramatic, to the point that 1% additive gave an excessively long separation.

Figs. 4 and 5 illustrate another consideration in these separations. Leucine-benzyl esters were purchased as tosylate salts. The tosylate counterion may interfere with the enantiomers and its elution needs to be considered. This peak is affected by modifier selection and the presence of amine additive. In Fig. 4, the tosylate peak elutes close to the (D) enantiomer when ethanol is used as modifier. With methanol modifier (Fig. 5) tosylate is separated from the analyte peaks, giving a more useful separation even though enantiomeric resolution is less. Fig. 6 illustrates the lack of amine memory effect observed in SFC. The chromatogram from Fig. 5 is overlaid with one generated immediately after the additive is observed to be clear from the mobile phase (UV background drop). After elimination of additive from the mobile phase the Leucine-benzyl ester enantiomers and the tosylate counter ion collapse to a single peak.

Two additional probes were subjected to further examination. The simple amine, α-methylbenzylamine, gave

Table 5
The effect of different modifiers and cyclohexylamine additive levels on the retention of Leucine–Benzyl ester

Modifier	% CHA	k'_1	k'_2	α	Rs
Methanol	0	0.74	0.74	1.00	0
	1.0	0.77	1.22	1.58	3.73
Ethanol	0	0.80	0.80	1.00	0
	0.1	0.82	1.14	1.38	2.00
	0.4	1.12	2.66	2.37	7.27
	1.0	1.39	4.28	3.08	10.8
2-Propanol	0	1.01	1.01	1.00	0
	0.1	1.13	2.96	2.63	4.28
	0.4	2.18	8.85	4.05	11.1
	1.0	3.62	14.07	3.88	15.7

Rs: Resolution calculated by Berger software; % CHA: vol.% cyclohexylamine in modifier; modifier at 20%, 2 ml/min, 180 bar, ambient temperature.

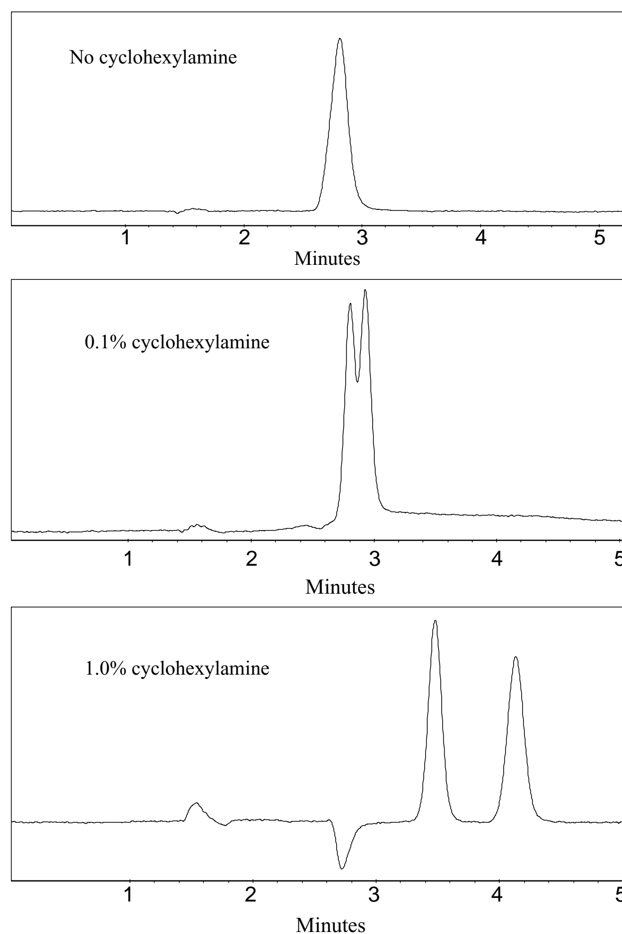

Fig. 2. SFC chromatograms of phenylalanine-methyl ester on an AD column with cyclohexylamine at various levels in ethanol modifier (20%). Flow rate is 2.0 ml/min with a back pressure of 180 bar, at ambient temperature.

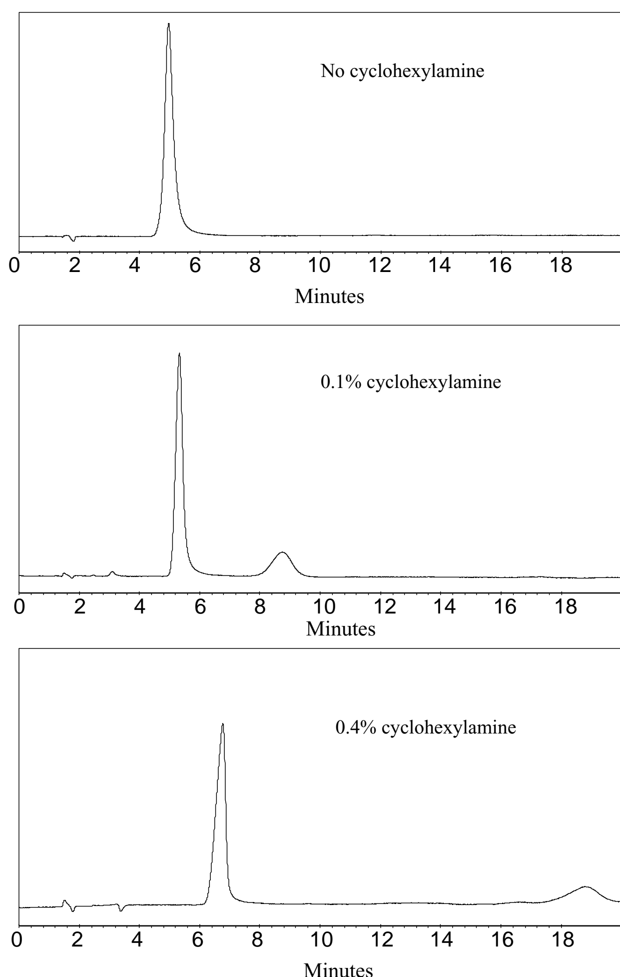

Fig. 3. SFC chromatograms of tyrosine-methyl ester on an AD column with cyclohexylamine at various levels in 2-propanol modifier (20%). Flow rate is 2.0 ml/min with a back pressure of 180 bar, at ambient temperature.

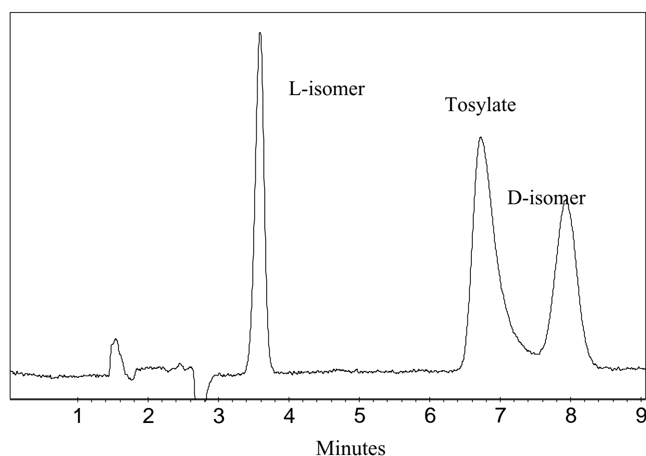

Fig. 4. SFC chromatogram of leucine-benzyl ester on an AD column with 1% cyclohexylamine in ethanol modifier (20%). Flow rate is 2.0 ml/min with a back pressure of 180 bar, at ambient temperature.

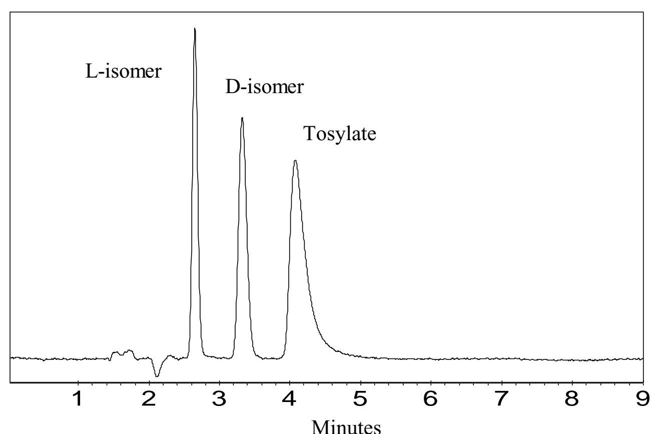

Fig. 5. SFC chromatogram of leucine-benzyl ester on an AD column with 1% cyclohexylamine in methanol modifier (20%). Flow rate is 2.0 ml/min with a back pressure of 180 bar, at ambient temperature.

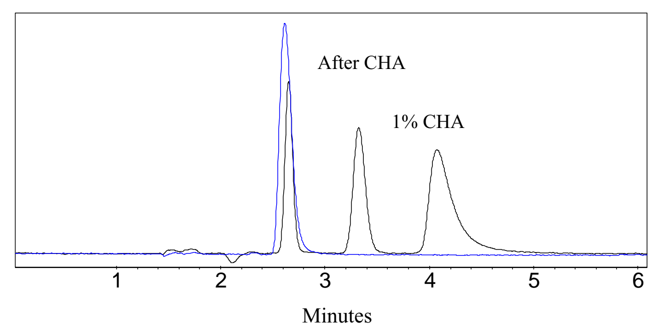

Fig. 6. Overlaid SFC chromatograms of leucine-benzyl ester on an AD column with 1% cyclohexylamine and immediately after its removal. The chromatogram identified as 1% CHA shows the L-isomer, the D-isomer and the tosylate counter ion (conditions as in Fig. 5). After CHA shows the immediate collapse to a single peak upon removal of the additive.

some separation in screening experiments. The amino alcohol, 2-amino-3-phenyl-1-propanol, gave indications of selectivity but nothing useful. Cyclohexylamine was added to 2-propanol modifier at levels up to 2% by volume. 2-Propanol was selected as being most likely to give favorable results. Results are presented in Table 6. Increasing additive level increased retention, selectivity and resolution for both probes.

Table 6
The effect of different cyclohexylamine additive levels on the retention of α-methylbenzylamine and 2-amino-3-phenyl-1-propanol

% CHA	α-Methylbenzylamine				2-Amino-3-phenyl-1-propanol			
	k'_1	k'_2	α	Rs	k'_1	k'_2	α	Rs
0	1.50	1.82	1.21	0.93	1.94	1.94	1.00	0.00
0.4	1.88	2.42	1.29	2.35	2.23	2.47	1.11	0.93
1.0	2.71	4.05	1.49	5.05	3.34	4.02	1.20	1.72
2.0	3.43	5.37	1.57	6.33	4.08	5.38	1.32	2.94

Rs: resolution calculated by Berger software; % CHA: vol.% cyclohexylamine in 2-propanol; 2-propanol at 20%, 2 ml/min, 180 bar, ambient temperature.

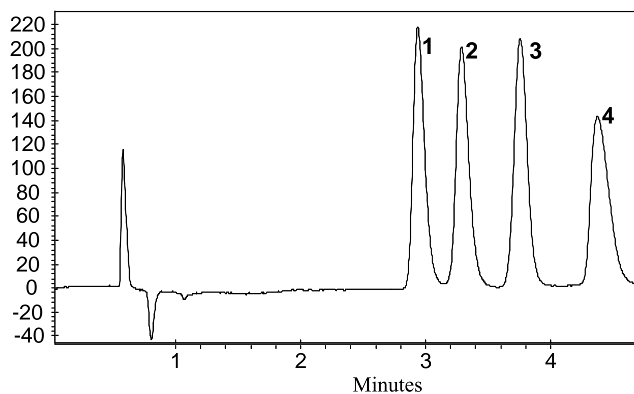

Fig. 7. SFC separation of amphetamine and methamphetamine enantiomers on a CHIRALPAK® AD-H® column using a 10% 2-propanol (0.5% cyclohexylamine) modifier at 5 ml/min; 150 bar. Peak **1** = (*S*)-methamphetamine, 2.94 min. Peak **2** = (*R*)-methamphetamine, 3.28 min; Peak **3** = (*S*)-amphetamine, 3.75 min; Peak **4** = (*R*)-amphetamine, 4.37 min.

The potential to separate small amine analytes suggests the possibility of analyzing amphetamine and methamphetamine (Fig. 1). The enantiomeric ratios of amphetamine and methamphetamine are affected by the means used to synthesize and isolate them. As such, these ratios are useful as a fingerprint, aiding in the identification of sources of illicit materials. Being controlled substances, these materials are available in limited supply and only in solution. A common observation in the chromatograms presented here is that of a vacancy peak. This may be attributed to a mismatch of additive amount in the sample injection and the amount in the mobile phase. With the supplied solutions of amphetamine and methamphetamine, manipulating the vacancy peak away from the analytes became a challenge. Tested additives included diethylamine, cyclopentyl, cyclohexyl and cycloheptyl amines. 2-Propanol, ethanol and methanol modifiers were examined as well as varying levels of additive. Conditions finally developed included the use of a CHIRALPAK® AD-H® column with 0.5% cyclohexylamine in 2-propanol modifier at 10%. An elevated flow rate was used to give baseline separation of all enantiomers of amphetamine and methamphetamine in 5 min (Fig. 7).

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