ELSEVIER

Journal of Chromatography A, 945 (2002) 139–146

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Memory effect of mobile phase additives in chiral separations on a Chiralpak AD column

Yun K. Ye, Barbara Lord, Rodger W. Stringham*

Dupont Pharmaceutical Company, Chemical Process Research and Development, Chambers Works, PRF Building (S1), Deepwater, NJ 08023-0999, USA

Received 20 August 2001; received in revised form 6 November 2001; accepted 7 November 2001

Abstract

Using chiral probes shown to be sensitive to the presence of mobile phase additives, a memory effect for these additives by an amylosic column was demonstrated. Exposure to these additives gave prolonged chromatographic performance changes even after their removal from the mobile phase. This finding is consistent with strong binding of the additives to the stationary phase. A procedure to remove bound additives was developed.

Keywords: Memory effect; Mobile phase composition; Enantiomer separation; Amino acids

1. Introduction

In recent work [1,2] it was demonstrated that acidic and basic mobile phase additives could be used to increase enantioselectivity in the chromatographic separation of underivatized amino acids on common amylosic columns. The effect of acidic additives appeared to be limited to amine analytes, and was attributed to either a minimization of non-specific retention arising from interaction with non-selective portions of the adsorbed polymer or silica support, or to an increased retention arising from interaction with bound additive. The effect of amine additives was attributed to a restriction of modifier access required to displace bound analytes. While it was not documented in these publications, early in development variable results were occasionally obtained. Variability was eventually traced to column history. A column used with mobile phase additives required extensive washing to return it to control or pre-additive behavior. This suggests a memory effect of the column induced by the use of additives.

Acidic and basic mobile phase additives are often used in chiral separations to minimize peak broadening arising from unwanted interactions between polar solutes and the stationary phase [3–8]. Although it is not reported in the literature there is ample anecdotal evidence that equilibration of amylosic columns is also slow for this application. Many researchers admit to dedicating columns to additive use once exposed, and there are vague allusions to a ''memory'' effect attributed to some undefined alteration in tertiary structure of the polymer. Closer study of this

*Corresponding author. Tel.: +1-856-540-4903; fax: +1-856-540-4902.

E-mail address: rodger.w.stringham@dupontpharma.com (R.W. Stringham).

0021-9673/02/$ – see front matter
PII: S0021-9673(01)01491-1

Table 1
Effect of ethanesulfonic acid on chiral separation of amino acid esters

Probe	ESA	k_1'	k_2'	α
Leucine, isobutyl ester	No	0.47	0.55	1.16
	Yes	0.47	0.79	1.67
Leucine, ethyl ester	No	0.52	0.61	1.17
	Yes	0.75	1.14	1.53
Leucine, methyl ester	No	0.69	0.77	1.12
	Yes	0.77	1.43	1.85
Phenylalanine, methyl ester	No	1.84	2.15	1.17
	Yes	1.40	4.12	2.95
4-Cl-Phenylalanine, methyl ester	No	2.34	2.54	1.09
	Yes	1.46	6.47	4.43
4-Cl-Phenylalanine, ethyl ester	No	1.82	1.82	1.00
	Yes	1.20	4.22	3.52
Tyrosine, methyl ester	No	5.90	5.90	1.00
	Yes	2.20	21.64	9.86

memory effect is confounded by the need to measure small changes in peak symmetry and efficiency in a system already showing poor peak symmetry and efficiency. The sometimes dramatic changes in selectivity observed recently [1,2] arising from the use of additives, should enable observation of the existence of a memory effect for additives on amylosic columns.

Table 1 presents the probe molecules selected for this study. These molecules are esters of amino acids leucine, tyrosine, phenylalanine and 4-chlorophenyalanine, with no derivatization of the amine functionality. Data in Table 1 were generated on a ChiralPak AD column using an ethanol–hexane mobile phase with and without ethanesulfonic acid (ESA). Incorporation of the acid resulted in increased k_2' values for all probes, with moderate to no increase in k_1' for the leucine esters and decreased $k'1$ for the phenyalanine and tyrosine esters. The net effect is a dramatic increase in enantioselectivity for all probes arising from the incorporation of ESA in the mobile phase. If there is a memory effect of the stationary phase for mobile phase additives it should be apparent in changes in this selectivity.

2. Materials and methods

2.1. Reagents

All reagents used in this study were reagent grade or better. High-performance liquid chromatography grade hexane was purchased from EM Sciences (Gibbstown, NJ). Absolute ethanol was obtained from Aaper Alcohol and Chemical Co. (Shelbyville, KY). All other reagents were obtained from Sigma–Aldrich (St. Louis, MO). β-blockers, phenylalanine, leucine and tyrosine analog samples were dissolved in ethanol with a final concentration about 1 mg/ml for phenylalanine, tyrosine analogs and the β-blockers and 5 mg/ml for leucine analogs.

2.2. Chromatography

Chromatographic studies were performed on an HP 1100 liquid chromatograph (Agilent, Palo Alto, CA) equipped with vacuum degasser, quaternary pump, autosampler, thermostatted-column device and a variable-wavelength UV detector. The chromatographic data were acquired and processed with computer-based Agilent Chemstation software. A ChiralPak AD column (250×4.6 mm, 10 μ) was purchased from Chiral Technologies, Inc. (Exton, PA) and was used as received. Unless otherwise noted, chromatographic studies were performed at 40 °C with a 1.0 ml/min flow-rate. The mobile phase consisted of 90 vol.% hexane and 10 vol.% of ethanol with or without different acidic and amine additives. After equilibrium had been achieved, 5-μl of sample solution was injected. Detection was achieved at 210 nm. Dead time was estimated by the retention time of the first solvent disturbance peak.

3. Results and discussion

3.1. Persistence of acidic additive effects

To evaluate the existence of a memory effect of the AD stationary phase for the ESA additive, the column was equilibrated with the mobile phase containing additive and then switched to the same mobile phase without ESA. Injections of the probe molecules were made at intervals for as long as 51 h flushing without additive. Results are shown in Figs. 1–7. In all cases the enhanced enantioselectivity persists with only slight declines for as long as 50 h after the discontinuation of additive from the mobile phase. The memory effect persists for at least 1000 column volumes of mobile phase, suggesting that the additive has become firmly bound to the stationary phase. There also appears to be small initial changes in retention and selectivity between time zero and the first time point, which may reflect the loss of additive from the mobile phase (Fig. 8). The size of the initial changes indicates that the beneficial effects of ESA on these separations derive primarily from additive bound to the stationary phase rather than that in the mobile phase.

Trifluoroacetic acid (TFA) was also evaluated for the existence of a memory effect. The methyl ester of tyrosine was chromatographed with 0.2% TFA giving an increase in selectivity from 1.00 to 2.32. Upon discontinuation of additive, selectivity declines from 2.32 to 1.58 within the first hour. Selectivity returns to 1.00 after 10 h of mobile phase flushing. Fig. 9 shows the comparison between the memory for TFA and ESA. The TFA enhancement of selectivity persists, but not as long as the ESA effect.

The dramatic persistence of the ESA effects after its removal from the mobile phase suggests that it is being strongly bound to the stationary phase, and

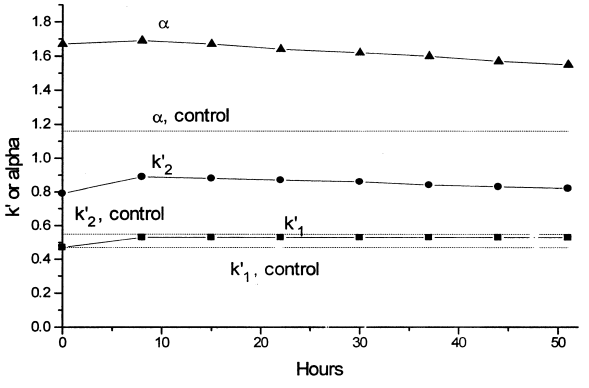

Fig. 1. Persistence of the effect of ethanesulfonic acid on the retention and selectivity of leucine, isobutyl ester on an AD column. Hours is the time after the discontinuation of ESA in the mobile phase. Dashed control lines represent the retention and selectivity before ESA was added to the mobile phase.

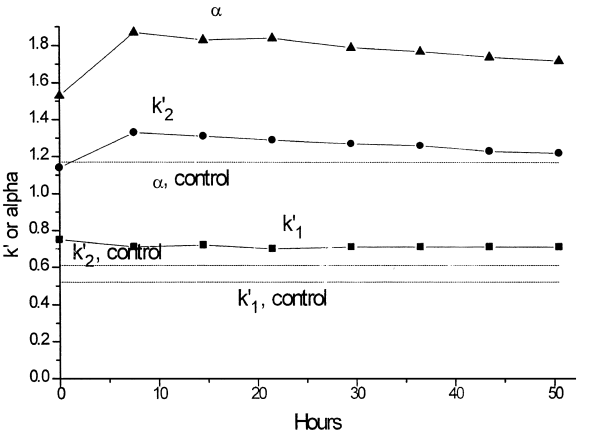

Fig. 2. Persistence of the effect of ethanesulfonic acid on the retention and selectivity of leucine, ethyl ester on an AD column. Hours is the time after the discontinuation of ESA in the mobile phase. Dashed control lines represent the retention and selectivity before ESA was added to the mobile phase.

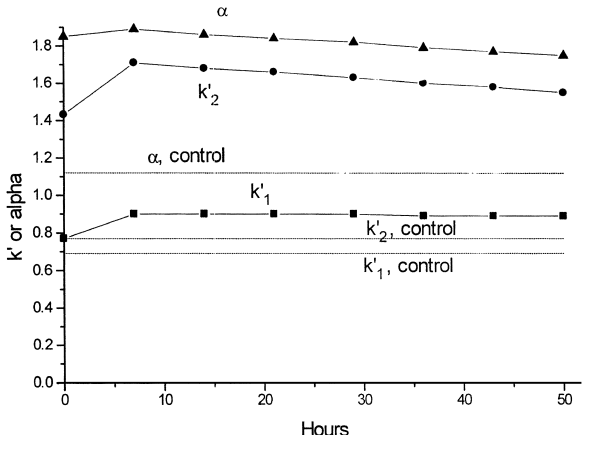

Fig. 3. Persistence of the effect of ethanesulfonic acid on the retention and selectivity of leucine, methyl ester on an AD column. Hours is the time after the discontinuation of ESA in the mobile phase. Dashed control lines represent the retention and selectivity before ESA was added to the mobile phase.

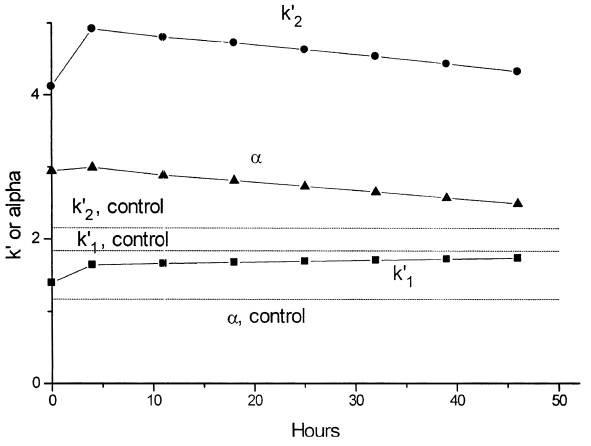

Fig. 4. Persistence of the effect of ethanesulfonic acid on the retention and selectivity of phenylalanine, methyl ester on an AD column. Hours is the time after the discontinuation of ESA in the mobile phase. Dashed control lines represent the retention and selectivity before ESA was added to the mobile phase.

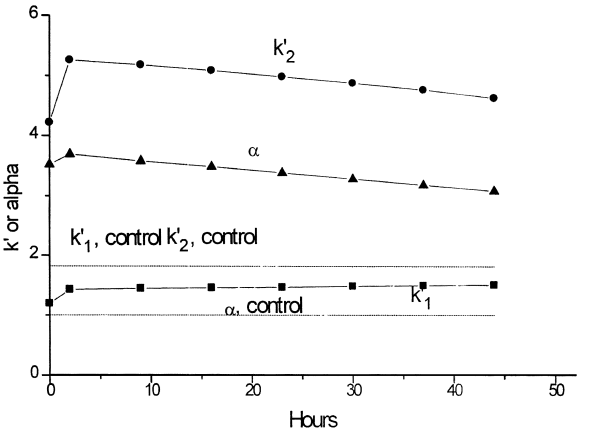

Fig. 6. Persistence of the effect of ethanesulfonic acid on the retention and selectivity of 4-chlorophenylalanine, ethyl ester on an AD column. Hours is the time after the discontinuation of ESA in the mobile phase. Dashed control lines represent the retention and selectivity before ESA was added to the mobile phase.

that the bound additive is the major source of the enhanced enatioselectivity. Since the additive need not be present in the mobile phase it may be possible to load it onto the column through injection. This should allow the use of lower amounts of additive and allow the use of fewer mobile phase reservoirs. The column was equilibrated with a (15:85) ethanol–hexane mobile phase and the tyrosine methyl ester mixture was injected. Subsequent to this a 100 μl injection of a 10 mg/ml ESA in (15:85) ethanol–hexane solution was made. After 5 min the tyrosine methyl ester mixture was injected. A sequence of such alternating injections was made until 19 mg of ESA had been loaded. Results are shown in Fig. 10. Enantioselectivity and k_2' increased while k_1' decreased with injected ESA up to 11 mg. Above this amount no additional effect was observed. The maximum selectivity of 5.3 is lower than previously

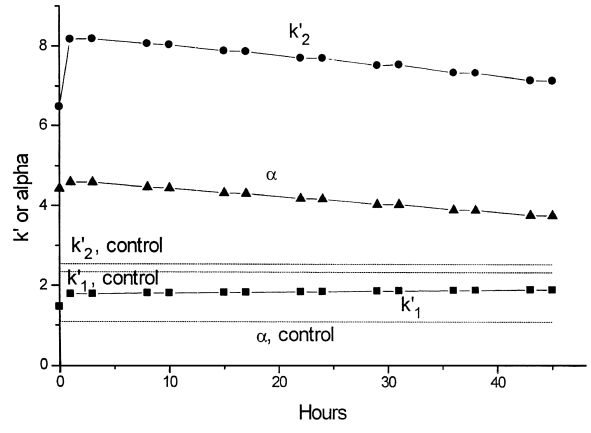

Fig. 5. Persistence of the effect of ethanesulfonic acid on the retention and selectivity of 4-chlorophenylalanine, methyl ester on an AD column. Hours is the time after the discontinuation of ESA in the mobile phase. Dashed control lines represent the retention and selectivity before ESA was added to the mobile phase.

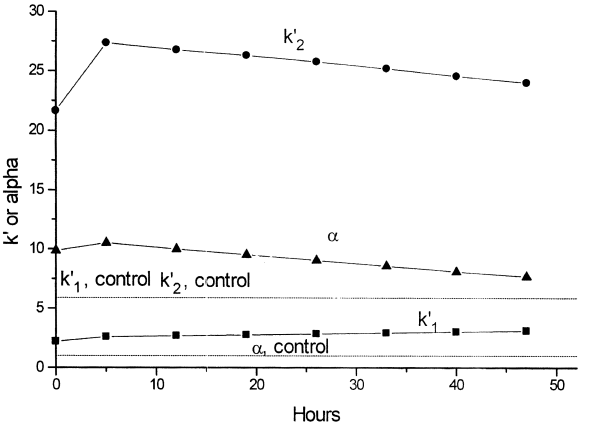

Fig. 7. Persistence of the effect of ethanesulfonic acid on the retention and selectivity of tyrosine, methyl ester on an AD column. Hours is the time after the discontinuation of ESA in the mobile phase. Dashed control lines represent the retention and selectivity before ESA was added to the mobile phase.

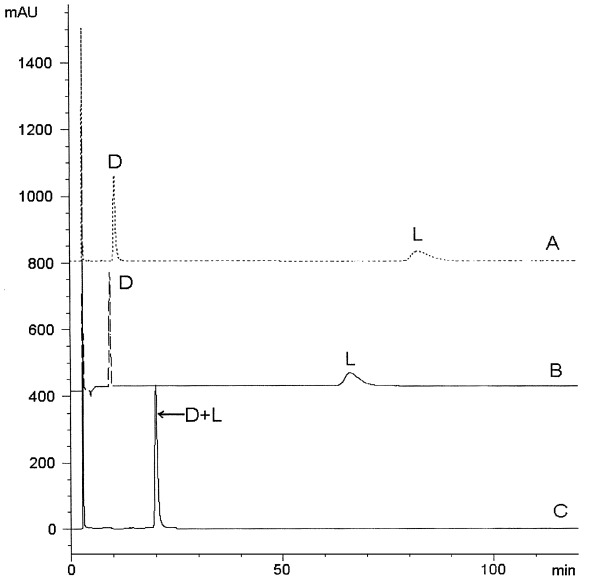

Fig. 8. Chromatograms of tyrosine–methyl ester without additive (C); with additive (B); and after removal of additive from the mobile phase (A).

observed. This was due to the higher ethanol levels used to speed the separation. At 10% ethanol the second peak elutes at 75 min. Enantioselectivity is a function of the amount of modifier used in the separation and is independent of the means of loading the additive on to the column.

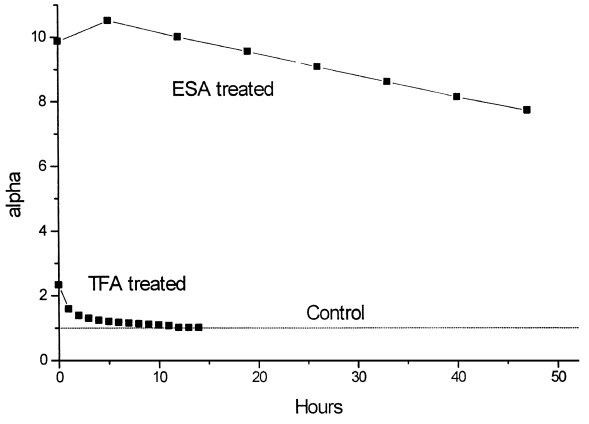

Fig. 9. Comparison of the persistence of the effects of ethanesulfonic acid and trifloroacetic acid on the enantioselectivity of tyrosine, methyl ester on an AD column. Hours is the time after the discontinuation of additive in the mobile phase. The dashed control line represents the selectivity before acid was added to the mobile phase.

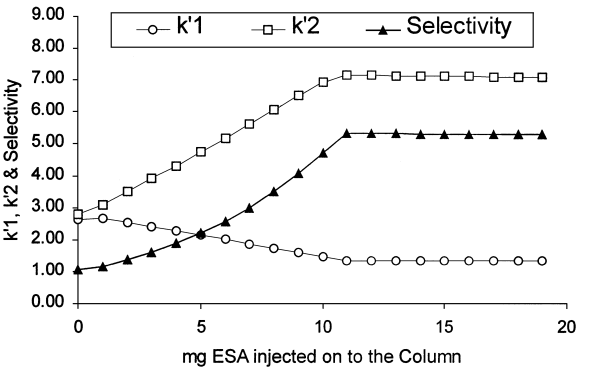

Fig. 10. The effect of loading of ethanesulfonic acid on retention and enantioselectivity of tyrosine methyl ester. A series of 1 mg ESA injections (100 μl injection of a 10 mg/ml ESA in (15:85) ethanol–hexane) was made, alternating with analyte injections.

3.2. Persistence of amine additive effects

It was found that for most probes evaluated, amine additive alone was insufficient to induce large changes in selectivity. There were some probes that showed moderate increases in enantioselectivity arising from the incorporation of amine additives. The addition of 0.2% butylamine to an 10% ethanol vs. hexane mobile phase gave approximately a 20% increase in selectivity for the separation of a series of β-blockers. When the additive was removed from the mobile phase, the beneficial effect persisted for at least 34 h (Table 2).

Large changes in selectivity could be observed when amine additives and acid additives were used together but no memory effect was observed. This suggested that the amine additives are solubilized and flushed out by the acid additive in the mobile phase. In an attempt to eliminate the effect of mobile phase ESA, the column was pre-treated with ESA and flushed with acid free mobile phase. When amine additive was incorporated in the mobile phase it was found that both amine and acid additive flushed from the column within 30 min.

3.3. Removal of bound additives

Data presented above clearly demonstrate that prior exposure to mobile phase additives will affect the performance of an amylosic column. Contrary to examples presented here this effect may not always

Table 2
Persistence of butylamine additive effect on the chiral separation of various β-blockers on an AD column

Analog	Time	k'_1	k'_2	Selectivity
Alprenolol	Before amine	0.40	0.51	1.27
Alprenolol	Original	0.39	0.59	1.52
Alprenolol	1-h mp rinse	0.41	0.61	1.49
Alprenolol	10-h mp rinse	0.40	0.60	1.49
Alprenolol	20-h mp rinse	0.40	0.60	1.50
Alprenolol	30-h mp rinse	0.40	0.60	1.50
Alprenolol	34-h mp rinse	0.40	0.60	1.50
Metoprolol	Before amine	1.62	1.86	1.14
Metoprolol	Original	1.56	2.07	1.32
Metoprolol	1-h mp rinse	1.66	2.15	1.29
Metoprolol	10-h mp rinse	1.64	2.13	1.29
Metoprolol	20-h mp rinse	1.65	2.13	1.29
Metoprolol	30-h mp rinse	1.65	2.13	1.30
Metoprolol	34-h mp rinse	1.65	2.13	1.30
Oxyprenolol	Before amine	0.71	0.91	1.29
Oxyprenolol	Original	0.72	1.11	1.54
Oxyprenolol	1-h mp rinse	0.76	1.15	1.52
Oxyprenolol	10-h mp rinse	0.75	1.14	1.52
Oxyprenolol	20-h mp rinse	0.75	1.14	1.52
Oxyprenolol	30-h mp rinse	0.75	1.14	1.52
Oxyprenolol	34-h mp rinse	0.75	1.14	1.52
Propranolol	Before amine	0.75	0.86	1.15
Propranolol	Original	0.84	1.13	1.35
Propranolol	1-h mp rinse	0.88	1.18	1.35
Propranolol	10-h mp rinse	0.87	1.17	1.35
Propranolol	20-h mp rinse	0.87	1.17	1.35
Propranolol	30-h mp rinse	0.86	1.16	1.35
Propranolol	34-h mp rinse	0.86	1.16	1.35

''Before amine'' represents results obtained with a 10% ethanol–hexane mobile phase. ''Original'' incorporates 0.2% butylamine into the mobile phase and ''rinse'' represents time after a return to initial mobile phase conditions.

be beneficial. Unless a chiral column can be dedicated to a single application, variable results will be observed depending on a column's history. It becomes vital to be able to control the effects of mobile phase additives. As demonstrated in Figs. 1–7 flushing with mobile phase does not remove additive. At the end of these experiements (after 50 h of flushing with mobile phase) the column was flushed for an additional 5 h with 100% isopropanol. This proved only partially successful in removing bound ESA (Table 3). It was ultimately found that water was required to remove bound ESA. After use columns are subjected to a sequence of isopropanol (to flush hexane) followed by 2 h of a 4:1 mix of isopropanol:water; then isopropanol; and ultimately back to mobile phase. This sequence served to restore the column to its original performance (Table 3). Amine additives required a flush with acid containing mobile phase (0.2% ESA in ethanol–hexane) prior to this sequence for restoration of original performance. We have used this flushing procedure on a several used columns that had been giving variable results in a particular application. Following flushing, consistent results were obtained across columns.

4. Conclusions

Data presented here clearly demonstrate the exist-

Table 3
Flushing of ethanesulfonic acid from an AD column

Probe	Flush[a]	k'_1	k'_2	α
Leucine, isobutyl ester	1	0.47	0.55	1.16
	2	0.47	0.79	1.67
	3	0.53	0.82	1.55
	4	0.56	0.81	1.44
	5	0.47	0.55	1.15
Leucine, ethyl ester	1	0.52	0.61	1.17
	2	0.75	1.14	1.53
	3	0.71	1.22	1.72
	4	0.74	1.18	1.59
	5	0.52	0.60	1.16
Leucine, methyl ester	1	0.69	0.77	1.12
	2	0.77	1.43	1.85
	3	0.89	1.55	1.75
	4	0.93	1.49	1.61
	5	0.69	0.76	1.11
Phenylalanine, methyl ester	1	1.84	2.15	1.17
	2	1.40	4.12	2.95
	3	1.74	4.34	2.50
	4	1.99	4.15	2.09
	5	1.90	2.23	1.18
4-Cl-Phenylalanine, methyl ester	1	2.34	2.54	1.09
	2	1.46	6.47	4.43
	3	1.90	7.16	3.77
	4	2.22	6.72	3.03
	5	2.37	2.59	1.09
4-Cl-Phenylalanine, ethyl ester	1	1.82	1.82	1.00
	2	1.20	4.22	3.52
	3	1.51	4.65	3.09
	4	1.72	4.32	2.52
	5	1.85	1.85	1.00
Tyrosine, methyl ester	1	5.90	5.90	1.00
	2	2.20	21.64	9.86
	3	3.12	24.15	7.73
	4	3.91	23.01	5.89
	5	6.29	6.29	1.00

[a] 1, prior to exposure to ESA; 2, with ESA; 3, 50 h flushing with mobile phase; 4, 5 h additional flushing with isopropanol; and 5, 2 h flush with 4:1 isopropanol–water.

ence of a memory effect of additives on an amylosic column, consistent with a binding of additive to the stationary phase. Removal of the additive from the mobile phase gives an initial change in the chromatography, followed by a lengthy persistence of the additive's effects. This suggests that additives may have different effects in the mobile phase and when bound to the stationary phase. Ethane sulfonic acid is more persistent as an additives than TFA. The effect of amine additives are less dramatic for the probes tested but these effects are no less persistent. A means of flushing bound additives from the stationary phase was developed and has been successfully applied to several columns of varying history.

The ability to reap the benefits of mobile phase additives without the need to continually supply them in the mobile phase will allow the use of less additive, will give less background absorbance, will allow the use of additives not soluble in the separation mobile phase and should enable preparative applications without the need to remove the additive from isolated fractions.

References

[1] Y.K. Ye, R.W. Stringham, J. Chromatogr. A 927 (2001) 47.
[2] Y.K. Ye, R.W. Stringham, J. Chromatogr. A 927 (2001) 53.
[3] Y. Okamato, M. Kawashima, R. Aburatani, K. Hatada, T. Nishiyama, M. Masuda, Chem. Lett. 1986 (1986) 1237.
[4] Y. Okamato, R. Aburatani, Y. Kaida, K. Hatada, Chem. Lett. 1988 (1988) 1125.
[5] Y. Okamato, R. Aburatani, K. Hatada, J. Chromatogr. 448 (1988) 454.
[6] A.M. Krstulovic, M.H. Fouchet, J.T. Burke, G. Gillet, A. Durand, J. Chromatogr. 452 (1988) 477.
[7] Y. Tang, Chirality 8 (1996) 136.
[8] Y. Tang, W. Zielinski, H. Bigott, Chirality 10 (1988) 364.