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Origin of enhanced chiral selectivity by acidic additives for a polysaccharide-based stationary phase

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Abstract

The effects of ethanesulfonic acid (ESA) and *n*-butylamine as additives were studied for a wide variety of chiral compounds using the polysaccharide chiral stationary phase (CSP), Chiralpak AD. The mobile phase consisted of hexane–ethanol (90:10, v/v). The additives typically had small effects, with one exception: the acidic additive had an enormous effect on the chiral selectivity of amino acid esters. The improved chiral selectivity was largely due to the longer retention of the later eluting enantiomer. Retention behavior of amines indicated that the higher selectivity for amino acid esters owes to increased hydrogen-bonding donation by the amine group of the analyte. Computation establishes the feasibility of a planar complex between the analyte and the cliral stationary phase, involving a pair of complementary hydrogen-bonding groups on each species, enabled by protonation of the analyte. Retention behaviors for a range of structures point to steric hindrance as the third interaction to comprise the requisite three interactions in chiral recognition.

Keywords: Chiral; Additives; Polysaccharide; Acidic; Stationary phase

1. Introduction

More than two hundred chiral stationary phases (CSPs) have been commercialised [1], since the first commercial CSP for HPLC that was introduced in 1981 [2]. However, one of the major problems for many commercially available CSPs is their limited applicability [3]. A narrow range of chiral selectivities was often observed for some of the small chiral selector CSPs, which usually require certain types of structures in the analytes to obtain satisfactory enantioseparation. A lack of physical and chemical stability and robustness were often found for protein CSPs. On the other hand, the polysaccharide-based CSPs, developed by Okamoto and co-workers [4–12], have proven to be among the most useful CSPs because of their versatility, durability, and in particular, their loadability for the preparative scale chromatography [13].

Since, most of the effort has been focused on the development of new polysaccharide-based CSPs in last decade, less effort has been devoted to investigate the effect of mobile phase variables, such as mobile phase modifiers and additives, which also play a crucial role in chiral selectivity. Since, the majority of the enantioseparation, using polysaccharide-based CSPs are operated under normal phase conditions with hexane-based mobile phases, the choice of modifiers are mainly limited to isopropanol or ethanol [14]. The use of mobile phase additives is quite routine in chiral HPLC employing polysaccharide-based CSPs under normal-phase conditions. In fact, up to a maximum of 1% (v/v) acidic and basic additives is frequently incorporated into mobile phase for polar analytes as a tailing reducer [15–21]. Severe tailing is often observed with compounds having amine groups, which require basic mobile phase additives, such as triethylamine

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and diethylamine, to be incorporated into the typical hexane/alcohol mobile phase to obtain satisfactory chiral selectivity [17–21].

For acidic compounds, acidic additives, such as trifluoroacetic acid and acetic acid, are required in any mobile phase combinations for the purpose of both efficiency and elution in normal phase mode [15,16]. The benefit of achiral acidic or basic additives is generally considered only to improve efficiency and peak shape for the polar analytes in chiral HPLC. Other important functions of the acidic and basic additives, such as effect on retention and selectivities, are overlooked for polysaccharide-based CSPs. In fact, improved selectivities were found in many cases when achiral acidic or basic mobile phase additives were used in mobile phase for chiral HPLC. Numerous studies have been reported in macrocyclic antibiotic-based [22–24], protein-based [25,26] and Pirkle type [27] of CSPs using achiral acidic and/or basic additives to control the chiral selectivity. Recent work [28–31] demonstrated that the acidic and basic additives could have a dramatic effect on both efficiency and chiral selectivity for amino acids using polysaccharide-based CSPs. However, a systematic study of the effect of additives in the chiral separation of a wide range of functionalities has not been done, despite the routine use of additives with polar analytes for polysaccharide-based CSPs [20,21,28–31]. Since, the chiral recognition mechanism at a molecular level on the polysaccharide-based chiral stationary phase, is still obscure [13], understanding the specific functions of acidic and basic mobile phase additives could also help to achieve the goal of elucidation of the chiral recognition mechanism for the polysaccharide-based CSPs. Such fundamental guidance would streamline methods development and reduce the trial and error approach.

In this work, the effect of the acidic acid additives ethanesulfonic acid (ESA) and basic additive butylamine (BA) on a broad range of chiral analytes is evaluated. ESA was chosen because it was the most effective acidic additive to improve both efficiency and selectivity based on the previous reported results [28–31]. For the same reason, BA was chosen as the basic additive for this study. The nature of the interactions between the stationary phase and additive were further investigated in a systematic manner to gain physical insight into the origin of chiral selectivity.

2. Experimental

All reagents used in this study were reagent grade or better. HPLC-grade hexane and isopropanol were purchased from EM Sciences (Gibbstown, NJ, USA); absolute ethanol was obtained from Aaper Alcohol and Chemical Co. (Shelbyville, KY, USA). All other reagents were obtained from Sigma–Aldrich (St. Louis, MO, USA). The individual enantiomer and racemic compounds studied were obtained from Sigma–Aldrich and Bachem (King of Prussia, PA, USA), and used without further purification. The structures of the studied compounds are shown in Fig. 1. The sample solutions were prepared in ethanol with a final concentration of about 1 mg/mL.

Chromatographic studies were performed on a HP 1100 liquid chromatograph (Agilent, Palo Alto, CA, USA) equipped with vacuum degasser, quaternary pump, autosampler, thermostatted-column device, and a variable-wavelength UV detector. The chromatographic data were acquired and processed with the computer-based Agilent Chemstation software. A Chiralpak AD column (250×4.6 mm, 10 μM) was purchased from Chiral Technologies (Exton, PA, USA), and was used as received. Fig. 2 represents the structure of the CSP. Unless otherwise noted, chromatographic studies were performed at 40 °C with a 1.0 mL/min flow rate. The mobile phase consisted of ethanol–hexane (10:90, v/v) with or without acidic and basic additives. After equilibrium had been achieved, 5 μL of sample solution was injected. Detection was achieved at 210 nm. Dead time t_0 was estimated by the retention time of the first solvent disturbance peak. The capacity factor and selectivity were calculated with their usual definitions of $k = (t_r - t_0)/t_0$ and $\alpha = k_2/k_1$, where k_1 and k_2 refer to the capacity factor for the first and second eluting enantiomer, respectively.

3. Results and discussion

Thirty chiral analytes, listed in Fig. 1, were chosen to have a broad range of functional groups to investigate the effect of acidic and basic additives. The chiral analytes are neutral compounds (**1**–**4** and **28**–**30**), acidic compounds (**6**–**14**), and basic compounds (**5**, **15**–**27**). Table 1 details the chromatographic retention behavior, with and without additives. All 30 tested analytes were eluted within a reasonable retention, except that no elution within 60 min was obtained with acidic analytes when no acidic additives were incorporated into mobile phase.

The effects of BA and ESA on chiral selectivity are dependent upon analyte functionality, as shown by the data in Table 1. For neutral compounds (analytes **1**–**4** and **28**–**30**), little change in retention and chiral selectivity was observed for either acidic or basic additives. For the acidic compounds (analytes **6**–**14**), elution was only observed when the acidic additive was present, but chiral selectivity was more significant when the analyte also had an amino group (analytes **13**, **14**). For the basic analytes (**15**–**18**), retention time was changed, but there was little change in chiral selectivity with acidic and basic additives. For the amino alcohol (analyte **5**), no chiral selectivity was obtained without additives, the presence of BA increased retention but gave no chiral selectivity, and the presence of ESA reduced retention but imparted chiral selectivity. For the amino acid esters (analytes **19**–**27**), while the basic additive has little effect on either retention or chiral selectivity, the acidic additive greatly enhances chiral selectivity. This inordinate enhancement in chiral selectivity for the amino acid methyl esters is interesting because of its

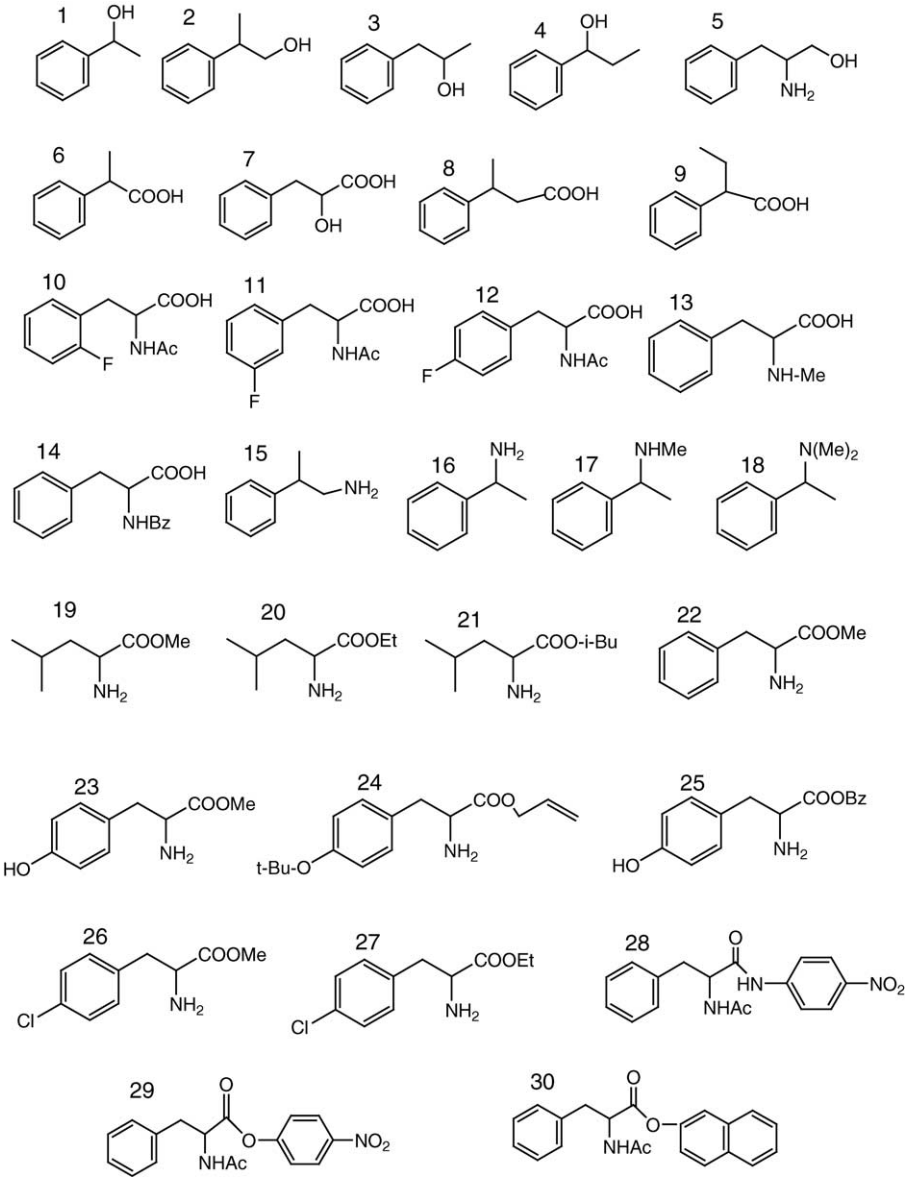

Fig. 1. Structures of analytes used in this study.

potential use in separations and because it is surprising in light of the only slight effect the additives have on resolution for the other compounds.

Fig. 3 shows the chromatograms obtained for an amino acid ester, analyte **24**, which undergoes the most dramatic increase in its chiral selectivity by ESA. The chromatograms show that when no additive was used, small chiral selectivity was obtained ($\alpha = 1.36$). After the basic additive, BA, was added, slightly decreased retention was observed but no improvement in selectivity was obtained. With the acidic additive ESA, inordinately improved selectivity was observed, whereby, there was little change in retention for the first eluted enantiomer but greatly increased retention for the second eluted enantiomer ($\alpha = 16.21$). The fact that only one enantiomer was strongly affected by the additive suggests that the recognition is rather uncomplicated, potentially allowing the nature of this recognition to be understood.

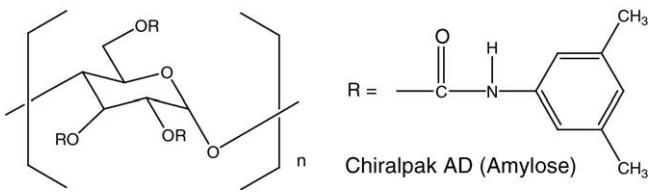

Fig. 2. Chiralpak AD chiral selector polymer structure.

Many of the observed trends in Table 1 can be plausibly explained. The negligible effects of acidic and basic additives on neutral compounds are not surprising, and this serves to indicate that the additives do not significantly change the

Table 1
Chromatographic results obtained with 10% (v/v) ethanol in hexane with or without additives using Chiralpak AD CSP

Probe	Class	No additives			0.2% BA			0.2% ESA		
		k_1	k_2	α	k_1	k_2	α	k_1	k_2	α
1	Alcohol	0.63	0.68	1.08	0.64	0.69	1.09	0.59	0.64	1.09
2	Alcohol	0.70	0.83	1.19	0.71	0.88	1.25	0.67	0.79	1.18
3	Alcohol	0.64	0.67	1.05	0.65	0.65	1.00	0.61	0.64	1.05
4	Alcohol	0.60	0.66	1.10	0.61	0.67	1.09	0.57	0.63	1.10
5	Amino alcohol	1.72	1.72	1.00	1.84	1.84	1.00	1.41	1.54	1.10
6	Acid	neo	neo		neo	neo		0.53	0.55	1.04
7	Acid	neo	neo		neo	neo		1.34	1.49	1.12
8	Acid	neo	neo		neo	neo		0.50	0.50	1.00
9	acid	neo	neo		neo	neo		0.48	0.48	1.00
10	*N*-protected amino acid	neo	neo		neo	neo		1.89	1.99	1.05
11	*N*-protected amino acid	neo	neo		neo	neo		1.54	1.60	1.04
12	*N*-protected amino acid	neo	neo		neo	neo		1.45	1.82	1.25
13	*N*-protected amino acid	neo	neo		neo	neo		1.22	1.42	1.17
14	*N*-protected amino acid	neo	neo		neo	neo		2.08	2.52	1.21
15	1° Amine	0.83	0.95	1.14	0.79	0.90	1.14	1.09	1.20	1.10
16	1° Amine	0.64	0.64	1.00	0.60	0.60	1.00	1.02	1.02	1.00
17	2° Amine	0.32	0.32	1.00	0.28	0.28	1.00	1.42	1.55	1.09
18	3° Amine	0.12	0.12	1.00	0.08	0.08	1.00	2.62	2.62	1.00
19	Amino acid ester	0.69	0.77	1.12	0.62	0.69	1.11	0.77	1.43	1.85
20	Amino acid ester	0.52	0.61	1.17	0.54	0.54	1.00	0.74	1.14	1.53
21	Amino acid ester	0.47	0.55	1.16	0.44	0.52	1.17	0.47	0.79	1.67
22	Amino acid ester	1.84	2.15	1.17	1.78	2.14	1.20	1.40	4.12	2.95
23	Amino acid ester	5.90	5.90	1.00	5.89	6.25	1.06	2.20	21.64	9.86
24	Amino acid ester	0.38	0.52	1.36	0.37	0.51	1.38	0.28	4.53	16.21
25	Amino acid ester	1.00	1.00	1.00	0.99	0.99	1.00	0.61	1.22	2.00
26	Amino acid ester	2.34	2.54	1.09	2.17	2.38	1.10	1.46	6.47	4.43
27	Amino acid ester	1.82	1.82	1.00	1.72	1.72	1.00	1.20	4.22	3.52
28	*N*-protected amino acidester	2.92	4.55	1.56	2.90	4.49	1.55	2.95	4.60	1.56
29	*N*-protected amino acid ester	5.34	60.66	11.35	5.33	60.50	11.35	5.35	60.80	11.36
30	*N*-protected amino acid ester	13.83	21.40	1.55	13.80	21.00	1.52	13.90	21.60	1.55

Note: neo = no elution observed (within 60 min). *Conditions*: mobile phase, 10% (v/v) EtOH in hexane; no additive, no additive added; BA, 0.2% (v/v) BA added; ESA, 0.2% (v/v) ESA added; column, Chiralpak AD CSP, 250 × 4.6 mm, 10 μ; flow rate, 1.0 mL/min; detection, UV at 205 nm; column temperature, 40 °C; injection volume, 5 μL. Other than noted, the chromatographic conditions will be same for all the table and figures.

structure of the CSP. The effect of the ESA in significantly shortening retention of acidic analytes could be due to acidic analytes interacting with the many residual aminopropylsilane groups on the silica surface used for adsorbing the CSP.

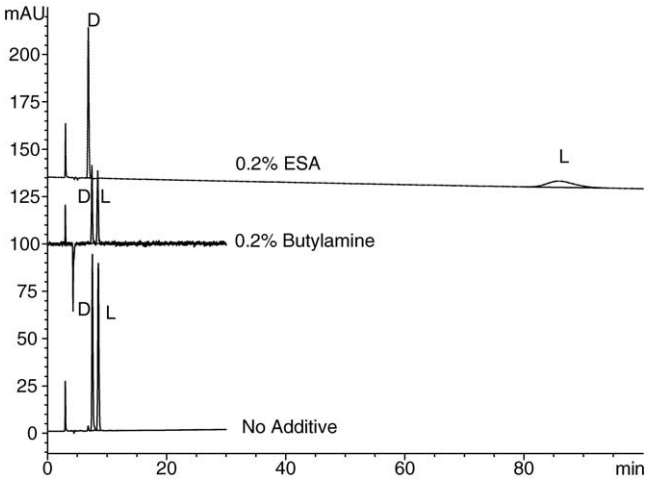

Fig. 3. Chromatograms obtained for analyte **24**. Chromatographic conditions are same as noted in Table 1.

The acidic additive would effectively compete for these sites on the aminopropylsilane groups by virtue of this higher concentration. The effect of BA in slightly shortening the retention of the amines can be attributed to the achiral basic additive simply competing with the basic analytes for whatever the adsorption sites are for these functional groups.

The effect of ESA in increasing retention time of bases is important because the basic functionalities are a component of the amino acid esters that are so strongly affected by ESA, therefore, the bases can lend valuable insight. An explanation for why ESA increases the retention time for the amines can be gained from trends in retention versus basicity. Fig. 4 shows that in the absence of ESA, retention times of the amines increase with hydrogen-bonding accepting ability, not donating ability. This suggests that the adsorption sites are amide groups on the CSP: basic amines would adsorb to the hydrogen-bond donating groups, the N–H groups, of the CSP. Fig. 4 shows that the opposite is true when ESA is added, indicating that ESA changes the retention mechanism, giving retention that owes to hydrogen-bonding donating ability. The moiety on the CSP that is a good hydrogen-bonding acceptor is the carbonyl group of the carbamate, therefore, it can be inferred that when the ESA protonates the amines,

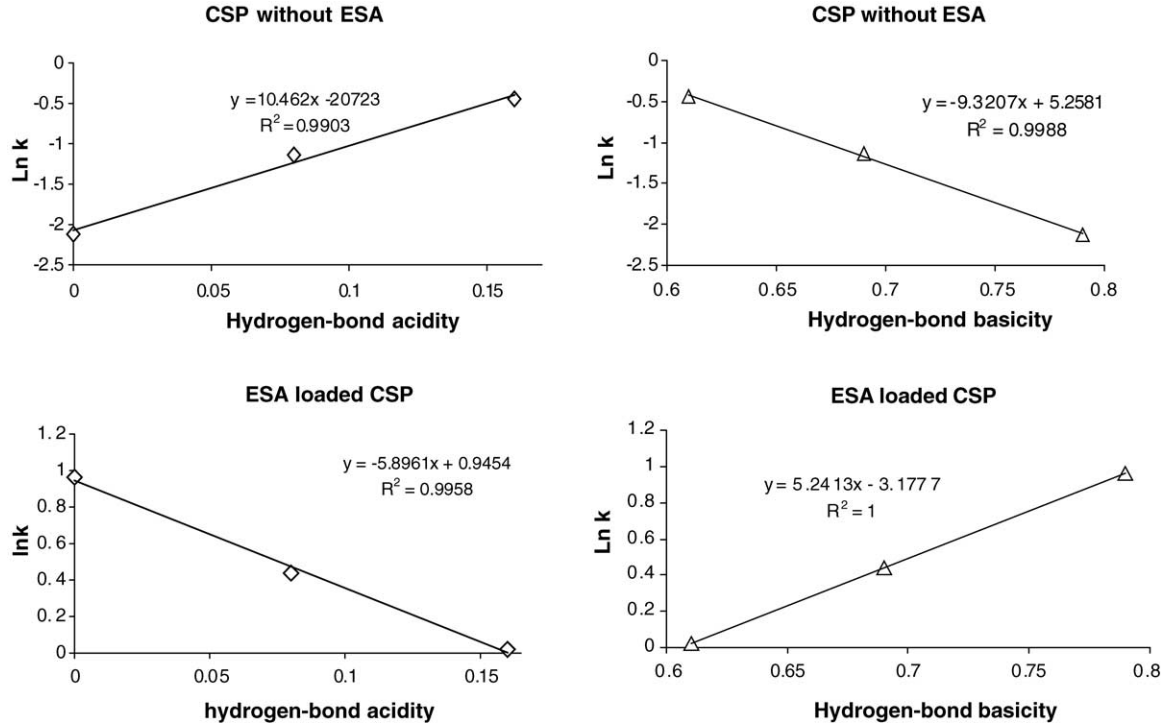

Fig. 4. Relationship between retention and hydrogen bond acidity and basicity of probes 16–18.

these analytes now adsorb to the carbonyl groups of the CSP. The overall increased retention times with addition of ESA indicate that interaction of protonated amines with CSP carbonyl groups is a stronger than the interaction of basic amines with the CSP N–H groups, which is not surprising. The low chiral selectivity for the amines relative to the amino acid esters indicates that the ester functionality, presumably the C=O group, plays a key role in chiral selectivity with this CSP.

The effect of the achiral acidic additive ESA in dramatically enhancing chiral selectivity of amino acid esters might be explained by these analytes having two hydrogen-bonding groups complementary to the two hydrogen-bonding groups of the CSP: the protonated amino group and the carbonyl group of the analyte form hydrogen bonds, respectively, to the carbonyl and amide groups of the CSP. This explanation requires that the groups could undergo these complementary hydrogen-bonding interactions without strain of the bonds, which is an idea that can readily be tested by computational chemistry. Fig. 5 illustrates a carbamate group, representing a section of the CSP that is hydrogen bonding to an amino acid ester, where the geometry of the complex was computed by energy minimization using the semi-empirical AM1 model. Ethyl groups are used to avoid cluttering the illustration and needlessly adding to the computational time. The figure shows that these two pairs of hydrogen-bonding interactions indeed create a planar 8-membered ring without strain, with the oxygen of the carbamate carbonyl nestled between two protons of the analyte's amino group. The two simultaneous interactions would explain a significant increase in retention time for the selected enantiomer. The achiral glycine methyl ester was used as the analyte to allow visualization of both enantiomers of a chiral amino acid, where the positions of amino acid side chains are labeled as **a** and **b** for the two enantiomers. If the requisite third interaction involved steric hindrance to block the amino acid side chain from being directed along **b** but not **a** (or vice versa), there would be chiral selectivity because only one enantiomer could achieve the strong, double hydrogen-bonding interaction. This would explain a large increase in retention for one enantiomer yet a negligible increase for the other. This simple idea is useful for

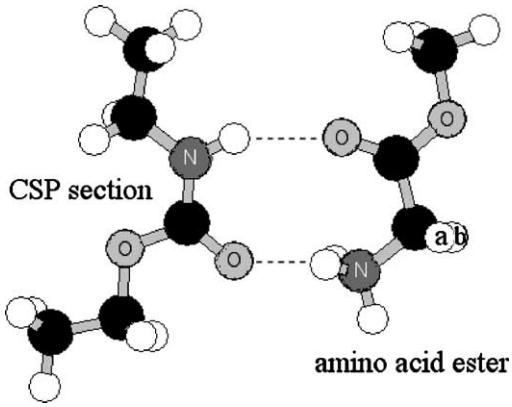

Fig. 5. Energy-minimized structure of doubly hydrogen bonded complex between a carbamate group of the chiral stationary phase (CSP) and an amino acid ester form a planar, doubly hydrogen bonded complex. (●) carbon, (○) hydrogen (◎) oxygen, (●) nitrogen. The labels **a** and **b** indicate the two directions of the amino acid side group for the two enantiomers.

illustration, and it is recognized that a quantitative explanation will involve consideration of the structure of the CSP, as well as dipole–dipole and π–π^* interactions [12,13,32,33].

The fact that only one enantiomer has its retention time increased, and the other enantiomer is virtually unaffected by the ESA, is remarkable, suggesting that the chiral recognition site is extraordinarily well structured. This seems rather dubious because the polysaccharide chains are flexible and the polysaccharide material is deposited without an effort to crystallize it onto the substrate, therefore, one would expect a continuum of recognition sites. It could be that there is some other contribution to retention that is reduced upon protonating the analyte, such as a hydrophobic interaction that requires the compound to be neutral. If reduction of a non-selective interaction offset the increased interaction of the non-selected enantiomer, then the recognition process would appear to be more selective than it is. The idea that the adsorption of less retained enantiomer is unaffected by ESA can be tested by Van Deemter plots, where the *C*-term contains information about the desorption time of the analyte from the stationary phase. *C* is related to desorption time, τ, as follows. Using *R* as the retention ratio, and neglecting other contributions to *C*, f_i, is the fraction of each type of:

$$C = \sum_i f_i R_i (1 - R_i)\tau_i$$

adsorption site. Double hydrogen bonding would be expected to increase the desorption time from the CSP because the activation barrier for desorption would be contributed by two different strong interactions. If the less retained enantiomer adsorbed to a significant number of sites allowing doubly hydrogen-bonded complexes, then it would also have a significantly increased *C* term, relative to the case of no ESA added. Conversely, since Table 1 shows that *R* is unchanged by ESA for the less retained enantiomer, then the *C* term ought to remain the same if ESA does not affect the adsorption of the lesser retained enantiomer.

Van Deemter plots for several of the amino acid esters were studied, with consistent results. Fig. 6 shows the Van Deemter plot for analyte **24**, which shows the plate height for the two enantiomers as a function of flow rate, with and without ESA. The behavior reveals that ESA primarily affects the *C*-term for the more retained enantiomer. There is some increase in *C* for the less retained enantiomer, indicating some recognition sites allowing stronger interactions. The small increase in *C* with addition of ESA indicates that the protonated amino acid esters are able to hydrogen bond somewhat more strongly to the CSP, perhaps by stronger single hydrogen bonding on the protonated groups and perhaps also some double hydrogen bonding. Since *C* is not large, there are fewer sites and/or weaker interactions for the less retained enantiomer. Since, *C* is increased but retention time is not, this does require a corresponding decrease in adsorption to non-selective sites, but this is not a large part of the story. Overall, the results show that the apparent extraordinary selectivity imparted to the amino acid esters by ESA is mainly due to high selectivity imparted to the chiral recognition process, and to a lesser extent, to reduced non-selective interactions of both enantiomers.

The possible role of ion-pairing must be addressed in this system of low dielectric constant. The chloride ion would not be expected to form a stable ion-pair with the amino acid ester. The chromatograms for several amino acid methyl esters were studied, using HCl as the additive rather than ESA, to determine if acidity is the factor. Fig. 7 shows the chromatograms for analyte **24**, which indicate that the chiral selectivity is increased significantly upon addition of HCl. Comparable results were obtained for the other amino acid esters. The fact that the chiral selectivity is not as high as for ESA, despite both being strong acids, indicates that there is another factor operating. It is likely that the residual amino

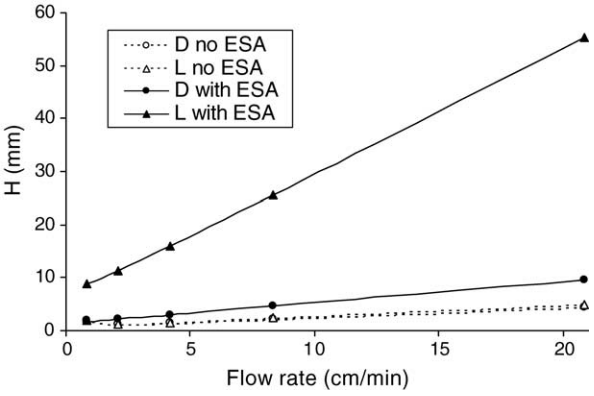

Fig. 6. Van Deemter plots for the L- and D-enantiomers of analyte **24**, with and without ethanesulfonic acid (ESA) added to the mobile phase.

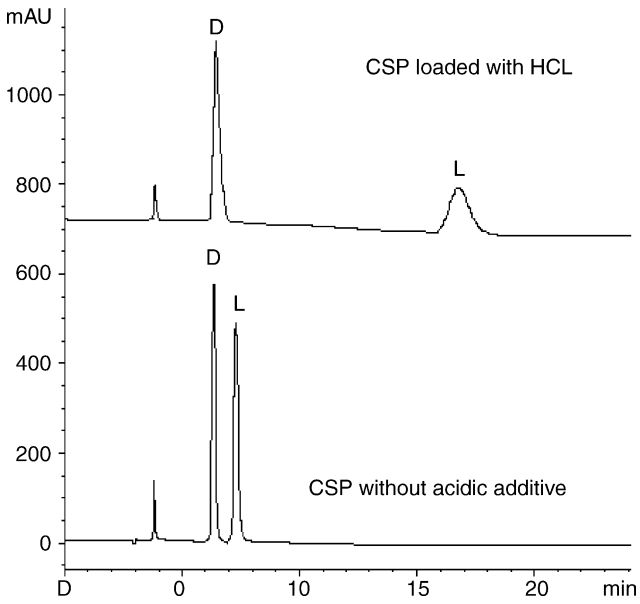

Fig. 7. Chromatograms obtained with and without hydrochloric acid for analyte **24**.

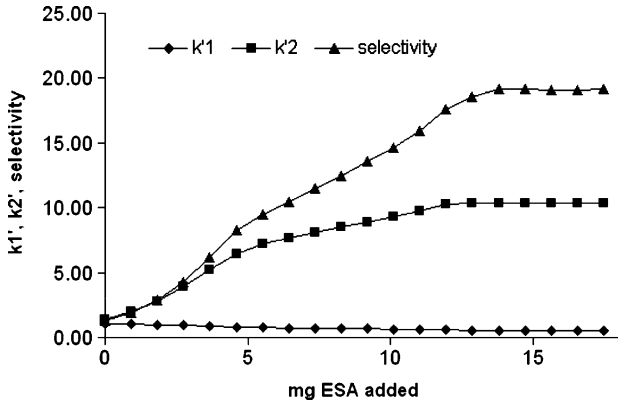

Fig. 8. Shift in retention with repeated injection of ESA for analyte (**24**).

groups on the chemically modified surface contribute to the local pH, requiring sufficient adsorption of acid to protonate the amino acid esters. Perhaps the surface is required to be adsorptive toward the acidic additive. This idea was tested by exposing the surface to the ESA additive over a period of time, and chromatograms were run during this treatment to determine if the retention behavior evolves. Fig. 8 shows that the chiral selectivity of analyte **24** increases with continued exposure to ESA, eventually leveling off. The ESA apparently accumulates on the surface. This behavior parallels that of the acid analytes, which do not elute in the absence of ESA. It is likely that organic acids, be they additives or analytes, accumulate on the surface by interaction with the residual amino groups of the aminopropylsilane used for adhesion of the CSP. The quantitative amount of ESA that accumulates on the surface corresponds to about 10% of the original amino groups, which supports the idea that acid is consumed in titrating the surface amino groups before the acidity is high enough to significantly protonate the analyte. The results support the structural model of double hydrogen bonding in a sterically constrained pocket to achieve chiral recognition of amino acid esters.

Among the compounds studied here, there are many other trends that can be explained by the structural model of Fig. 5. (1) Comparison of the methyl esters of phenylalanine (**22**; $\alpha = 2.95$) and isoleucine (**19**; $\alpha = 1.85$) shows that increased size of the hydrocarbon group increases selectivity, supporting the notion that steric hindrance is the third interaction. (2) By contrast, increased size on the ester group decreases selectivity, as seen by comparing methyl (**26**; $\alpha = 4.43$) and ethyl (**27**; $\alpha = 3.52$) chlorophenylalanine esters. It is possible that the bulkier group puts strain on the planar complex. (3) Selectivity is less than half for the amino acid, phenylalanine (**10**; $\alpha = 1.37$) compared to its methyl ester (**22**; $\alpha = 2.95$). The greater selectivity for amino acid esters than for amino acids, both the in presence of ESA, is consistent with Fig. 5 because the ester carbonyl is a better hydrogen bond acceptor for the donor NH_2 group of the CSP. (4) There is one notable exception to the rules here: analyte 12 behaves as though its amide group becomes protonated. The higher selectivity might be explained at least in part by the steric hindrance from the fluorine group being in the *para* position.

Overall, the set of readily available compounds used in this survey points to there being a tractable explanation for the observed effects of ESA on chiral selectivity. Future work with a set of systematically designed chiral compounds, combining chromatography and computation, is needed to support more directly the structure depicted in Fig. 5 and to provide a quantitative description of the chiral recognition for this family of compounds and this CSP.

Acknowledgement

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