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Structural characteristics of low-molecular-mass displacers for cation-exchange chromatography
II. Role of the stationary phase

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Abstract

The relative efficacy of a variety of low-molecular-mass displacers was examined on three different stationary phase materials. Several homologous series of displacer molecules were evaluated on these ion- exchange resins using a displacer ranking plot based on the steric mass action model. The results demonstrate that while aromaticity and hydrophobicity can play a significant role in the affinity of displacer molecules on polymethacrylate based and hydrophilized polystyrene–divinylbenzene based materials, this effect is much less pronounced on an agarose based resin. The work presented in this paper demonstrates that different structural features of low-molecular-mass displacers can dominate their affinity on various stationary phase materials employed and provides rules of thumb for the design of high affinity, low-molecular-mass displacers for a variety of commercial cation-exchange materials.

Keywords: Displacers; Steric mass action model; Stationary phases, LC; Amines

1. Introduction

The design of efficient downstream processes for the large-scale purification of biomolecules from complex biological mixtures continues to be one of the major challenges facing the biotechnology industry. In recent years, displacement chromatography has attracted significant attention as a promising preparative technique for protein separations [1–5].

However, to date, not much attention has been paid to the design of displacer molecules or to their efficacy on different classes of ion-exchange stationary phase materials. Conventionally, large polyelectrolytes have been employed as displacers for ion-exchange systems [6–9]. The basis for that choice was the assumption that a larger number of charges was required for a molecule to be able to displace proteins from ion-exchange resins. Recently it has been established that low-molecular-mass (<2000) compounds with a small number of charges can be used as displacers for proteins [10]. Several classes of low-molecular-mass displacers have been identified including dendrimers [11], protected amino acids [10] and antibiotics [12]. While these molecules have been successfully employed for protein purification in ion-exchange systems, they possessed moderate affinities and have thus been unable to displace highly retained biomolecules. There is thus a significant driving force for the development of

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high affinity, low-molecular-mass displacers for ion-exchange chromatography.

While it has been recognized that retention in ion-exchange systems is not purely based on electrostatics [13–15], there are only a few reports in the literature concerning the relative importance of non-specific interactions in governing affinity in ion-exchange materials [16]. The introduction of aromatic and alkyl functionalities has been found to increase retention on polystyrene–divinylbenzene (unhydrophilized) supports [17–23]. However, commercial polystyrene–divinylbenzene (PS–DVB) supports employed for protein chromatography are typically coated with a hydrophilic polymer to reduce irreversible protein adsorption [24]. The presence of this hydrophilic coating is expected to considerably alter the retention properties of this resin for various solutes. McNeff and Carr [25] investigated the hydrophobic character of polyethyleneimine coated zirconia anion exchangers and Kril and Fung [26] observed the contribution of aromaticity to the retention of singly charged primary and secondary amines on an Amberlite resin. However, to date there has not been a detailed comparison of the differences in the molecular features that contribute to the retention process in different classes of ion-exchangers possessing different backbone chemistries.

A systematic study of the structural features of small molecules which govern their retention on various ion-exchange stationary phases is critical for the design of high affinity, low-molecular-mass displacers capable of displacing highly retained biomolecules on these materials. Recent research has examined the affinity of homologous series of cationic molecules [27] on a polymethacrylate (PMA) based stationary phase. That study on a single resin indicated the importance of hydrophobic/aromatic interactions in governing retention.

In this paper, the relative efficacies of several homologous series of low-molecular-mass displacers are examined on three classes of stationary phases which are often employed for protein chromatography: a polymethacrylate based stationary phase (Waters SP-8HR), an agarose based resin (SP Sepharose) and a hydrophilized PS–DVB based stationary phase (Poros HS50). This study provides insight into the effects of various structural features on displacer affinity in relation to stationary phase chemistry.

2. Theory

The steric mass action (SMA) model [28] has been shown to successfully predict complex behavior in ion-exchange systems. The model involves three parameters for each solute: the characteristic charge (ν) which is the average number of sites that a molecule interacts with on the surface; the steric factor (σ) which is the average number of sites on the surface which are sterically shielded by the molecule and the equilibrium constant (K) of the exchange reaction between the solute and the salt counter-ions on the surface.

The equation for the SMA isotherm [29] for a single component is given as:

$$K = \left(\frac{Q}{C}\right) \cdot \left(\frac{C_{salt}}{\Lambda - (\nu + \sigma)Q}\right)^{\nu} \quad (1)$$

where Q and C are the solute concentrations on the stationary and mobile phases, respectively, and C_{salt} is the mobile phase salt concentration. The SMA isotherm is an implicit isotherm which can successfully predict non-linear, multicomponent behavior over a range of mobile phase salt concentrations once the parameters (K, ν and σ) have been determined [30–33] as described below.

A stability analysis on a displacement train [29] leads to the following criterion for component A to precede component B in a displacement train:

$$\left(\frac{K_A}{\Delta}\right)^{\frac{1}{\nu_A}} < \left(\frac{K_B}{\Delta}\right)^{\frac{1}{\nu_B}} \quad (2)$$

where $\Delta = Q_d/C_d$ and Q_d and C_d are the displacer concentrations on the stationary phase and in the mobile phase, respectively. The combination of terms on the left hand side of Eq. (2) can be defined as the dynamic affinity (λ) of the component:

$$\lambda = \left(\frac{K}{\Delta}\right)^{\frac{1}{\nu}} \quad (3)$$

The dynamic affinity is a measure of the affinity of a solute in a displacement scenario and effectively determines if a solute has sufficient affinity to displace another under the given experimental conditions (which are reflected in the value of Δ). Thus the dynamic affinity of a displacer molecule is an effective measure of its efficacy as a displacer.

To enable the comparison of displacer efficacies

over a range of operating conditions, Eq. (3) is rearranged and the parameter (λ) is plotted against the operational parameter (Δ).

$$\log \lambda = \frac{1}{\nu}\log K - \frac{1}{\nu}\log \Delta \quad (4)$$

This displacer ranking plot [27] has been shown to be capable of comparing the efficacies of various displacers on a given stationary phase over a range of operating conditions. In this paper, the displacer ranking plot is used to rank various homologous series of molecules on different stationary phase materials. It should be noted that while this plot may be employed for comparisons on a given stationary phase; the efficacies cannot be quantitatively compared between different stationary phase materials since the SMA parameter K (equilibrium constant) depends on various factors such as the ionic capacity and the porosity of a given stationary phase material. Nevertheless, the trends in the relative affinities of various homologous series may be compared on various stationary phases and can lead to insight into the behavior of these different resin systems.

3. Experimental

3.1. Materials

A strong cation-exchange (SCX, Waters SP-8HR, 100×5 mm) column was obtained from Waters (Milford, MA, USA). Pharmacia high-performance SP Sepharose (Pharmacia, Uppsala, Sweden) and Poros HS50 (Perkin-Elmer, Framingham, MA, USA) stationary phases were slurry packed in 95×16 mm and 250×4.6 mm columns, respectively. A Zorbax C_3 reversed-phase column (250×4.6 mm) was obtained from BTR Separations (Wilmington, DE, USA). Sodium monobasic and dibasic phosphate, fluorescamine, spermine tetrahydrochloride and spermidine trihydrochloride were purchased from Sigma (St. Louis, MO, USA). Bromophenol blue and potassium nitrate were obtained from Fisher Scientific (Pittsburgh, PA, USA). Aniline·HCl, benzylamine·HCl, butylamine, methylamine·HCl, 1,4,8,11-tetrazacyclotetradecane, tetraethylenepentamine pentahydrochloride, triethylenetetramine tetrahydrochloride, diethylenetriamine trihydrochloride and bis(hexamethylene)triamine trihydrochloride were purchased from Aldrich (Milwaukee, WI, USA). PETMA4 [pentaerythrityl(trimethylammonium(4))], DPE-TMA6 [dipentaerythrityl-(trimethylammonium(6))], PhTMA6 [phenyl (trimethylammonium(6))] and PE-DMABzCl4 [pentaerythrityl (dimethylammonium, benzyl(4))chloride] were synthesized as described elsewhere [34]. DPE-DMABzCl6 [dipentaerythrityl (dimethylammonium, benzyl(6))chloride], PE-DMAHepI4 [pentaerythrityl (dimethylammonium, heptyl(4))iodide] and PE-DMACyI4 [pentaerythrityl (dimethylammonium, cyclohexyl methyl(4))iodide] were synthesized as described below.

3.2. Apparatus

Linear gradients were run on a Pharmacia fast protein liquid chromatographic (FPLC) system consisting of two P-500 pumps and an LCC-500 controller. When appropriate, the column effluent was monitored using a Spectroflow 757 absorbance detector (Kratos, Ramsey, NJ, USA) and the signal data acquired using a Maxima 820 chromatography workstation and a MILLENIUM 2010 chromatography workstation (both from Waters). Column effluent fractions during the linear gradient runs were collected using a RediFrac fraction collector (Pharmacia). Fluorescence measurements were carried out on a LS50B spectrofluorometer (Perkin-Elmer, Wilton, CT, USA). UV absorbances for samples were measured on a Lambda 6 UV–vis spectrophotometer (Perkin-Elmer).

3.3. Procedures

3.3.1. Determination of SMA parameters for displacers

All experiments were carried out at pH 6 using a 20 mM phosphate buffer. Varying concentrations of NaCl were added to adjust the Na^+ ion concentration in the mobile phase.

The characteristic charge (ν) was determined from the induced salt gradients produced during non-linear frontal experiments using 5–15 mM of the displacer. These experiments were carried out at low ionic strengths (20 mM Na^+ concentration) and on-line conductivity detection was employed to measure the salt ion concentrations during the experiment. The characteristic charge is given simply by the ratio of

the magnitude of the induced salt gradient to the displacer concentration [35].

The same frontal experiments also furnished the breakthrough volumes of the displacers and were used to calculate the steric factor (σ) as described elsewhere [35]. (note: once the characteristic charge (ν) and the equilibrium constant (K) have been obtained, the steric factor is the only unknown and can be directly determined from the SMA isotherm Eq. (1).

The equilibrium constant was obtained from the retention volumes of the displacers in linear gradient chromatography with four to five different gradient slopes. The expression relating the retention volume of a particular solute on a linear gradient is given by [34]:

$$V_g = \left(\left[x_i^{\nu+1} + \frac{V_m K \beta \Lambda^{\nu} (\nu+1)(x_f - x_i)}{V_G} \right) \right] \cdot \left(\frac{V_G}{x_f - x_i} \right) \quad (5)$$

where V_g is the retention volume of a solute in the linear gradient (corrected for the gradient dwell volume in the system), V_G is the total volume of the mobile phase for the gradient experiment, V_m is the column dead volume, x_i and x_f are the initial and final salt concentrations in the gradient, β is the column porosity and ν and K are the SMA parameters. This expression was employed to calculate the parameter K and also provided an independent measurement for the parameter ν.

The ionic capacity (Λ) of the stationary phases were determined by titration. Column volumes (5–10) of 0.1 *M* HCl, deionized water and 1 *M* KNO_3 were passed sequentially through the column. The column effluent was collected when 1 *M* KNO_3 was infused and was titrated against NaOH using phenolphthalein as an indicator. (note: a 0.1 *M* acetic acid solution was substituted for the 0.1 *M* HCl solution for the SP Sepharose stationary phase to stay within its stability limits). The ionic capacities were determined to be: 435.7, 424 and 435.9 mequiv./ml stationary phase volume for the Waters SP-8HR, SP Sepharose and Poros HS50 stationary phases, respectively.

3.3.2. Synthesis of displacers

The synthesis of PETMA4, DPE-TMA6, PhTMA6 and PE-DMABzCl4 have been described elsewhere [34]. The synthesis of DPE-DMABzCl6 follows a similar route to that employed for preparing PE-DMABzCl4 from PE-Br4 [34]. DPE-Br6 (obtained during synthesis of DPE-TMA6 [34]) was refluxed for 24 h with the potassium salt of *N,N* dimethylethanolamine in dimethylformamide (DMF) as shown in Fig. 1a. Quaternization of DPE-DMA4 with benzyl chloride in acetonitrile under reflux for 24 h, followed by precipitation in diethyl ether yielded DPE-DMABzCl6.

Fig. 1b shows the synthesis schemes for PE-DMACyI4 and PE-DMAHepI4. PE-DMA4 (obtained during synthesis of PETMA4 [34]) was quaternarized with cyclohexylmethyl iodide and heptyl iodide in gently refluxing ethanol. PE-DMACyI4 was recrystallized from cold ethanol and PE-DMAHepI4 was rubbed with ether to induce crystal formation.

Fig. 1c shows the synthesis scheme for PE-tetramine which was prepared according to Fleischer's method [36]. The OH groups of pentaerythritol were converted to benzenesulfonate groups by reaction with benzenesulfonyl chloride in pyridine. This benzenesulfonate derivative (PE-OBs4) was transformed to the tetraazide (PE-Az4) by treatment with sodium azide in diethylene glycol. After recrystallization from ether, the transparent crystals of pure tetrakis (azidomethyl) methane were reduced to the tetramine with lithium aluminum hydride in THF.

^{1}H-NMR and ^{13}C-NMR spectroscopy were found to be in agreement with the proposed structures. The results are listed below:

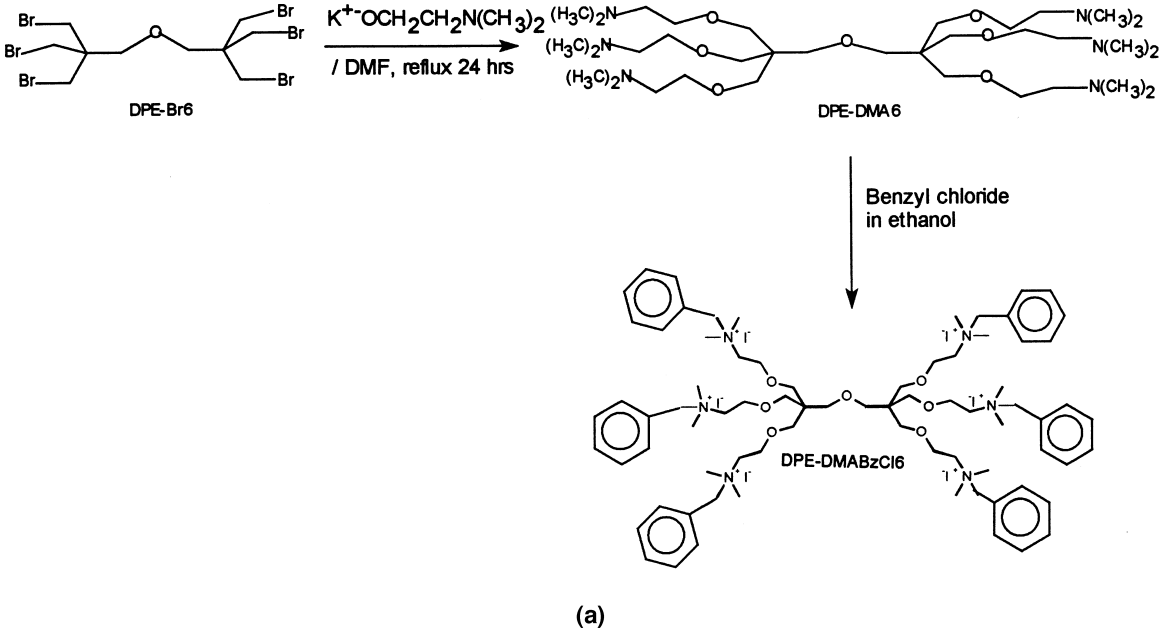
3.3.2.1. DPE-DMA6

^{1}H-NMR (C^2HCl_3, ppm) 2.16 (s, –N $(CH_3)_2$, 36H), 2.38 (t, $-CH_2-N-$, 12H), 3.24 (s, $O-CH_2-C-$, 4H), 3.29 (s, $C-CH_2-O-$, 12H), 3.38 (t, $-O-CH_2-CH_2-$, 12H). ^{13}C–NMR (C^2HCl_3, ppm) 45.17 (quaternary C), 45.39 ($-CH_2-$ N–), 45.79 ($-N(CH_3)_2$), 58.47 ($O-CH_2-C$), 69.96 ($C-CH_2-O-$), 70.03 ($-O-CH_2-CH_2-$). IR (neat): 2942, 2865, 2817, 2768, 1458, 1113, 1058, 1043 cm^{-1}.

3.3.2.2. DPE-DMABzCl6

^{1}H-NMR ([$^2H_6$]dimethyl sulfoxide, DMSO, ppm) 3.26 (s, –N $(CH_3)_3$, 54H), 3.38 (s, $O-CH_2-C$, 4H),

3.61(s, C–CH_2–O–, 12H), 3.84 (s, –CH_2–N, 12H), 4.04 (s, O–CH_2CH_2–, 12H), 5.08 (s, –CH_2–C_6H_5, 12H), 7.58 (m, aromatic, 18H), 7.81 (d, aromatic, 12H). ^{13}C–NMR ([$^2H_6$]DMSO, ppm) 44.82 (quaternary C), 49.65 (–N $(CH_3)_3$),62.93(–O–CH_2–CH_2–N–), 64.67, 66.(–O–CH_2–C–CH_2–O–), 128.50, 128.93, 130.32,133.35 (aromatic). IR (KBr): 3426, 2876, 1480, 1458, 1217, 1116, 769 and 717 cm^{-1}.



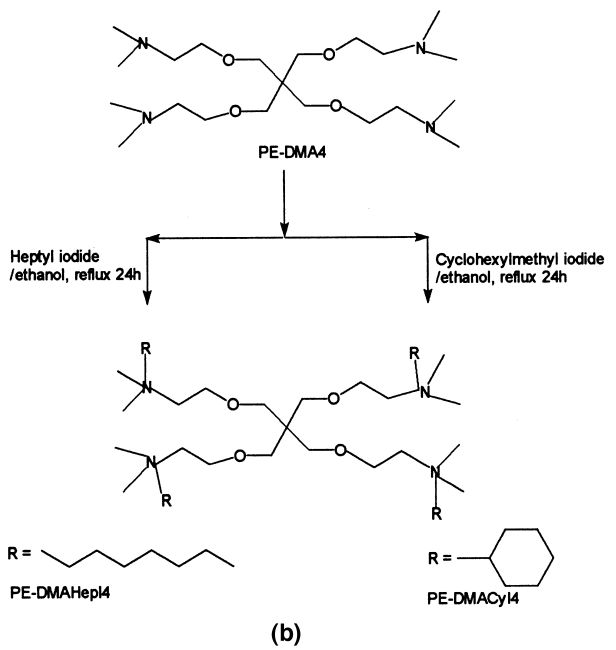


Fig. 1. Synthesis schemes for (a) DPE-DMABzCl6 from DPE-Br6, (b) PE-DMAHepI4 and PE-DMACyI4 from PE-DMA4 and (c) for PE-tetramine.

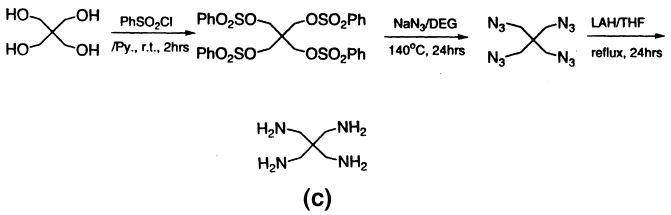


Fig. 1. (*continued*)

3.3.2.3. PE-DMACyMI4

^{1}H-NMR ([$^{2}H_6$]DMSO, ppm) 1.23, 1.48, 1.75, 1.92, 2.12 (–C_6H_{11}, 44H), 3.28(s, –N $(CH_3)_2$–, 24H), 3.45 (s, CH_2–C_6H_{11}, 8H), 3.55 (s, C–CH_2–O, 8H), 3.73 (s, –O–CH_2–CH_2–N, 8H), 3.92 (s, –O–CH_2–CH_2–N, 8H). ^{13}C- NMR ([$^{2}H_6$]DMSO, ppm) 25.17, 25.30, 31.14, 32.84,44.49 (quaternary C), 50.88 (N $(CH_3)_2$), 63.13, 64.62 (O–CH_2–CH_2–N), 70.02 (C–CH_2–O), 71.14 (–CH_2–C_6H_{11}). IR (KBr): 3425, 3007, 2965, 2877, 1481, 1456, 1119, 769, 718 cm^{-1}.

3.3.2.4. PE-DMAHepI4

^{1}H-NMR ([$^{2}H_6$]DMSO, ppm) 0.98 (t, –CH_3, 12H), 1.39 (bs, –CH_2–, 32H), 1.80 (s,N–CH_2–CH_2–, 8H), 3.22 (s,– N$(CH_3)_2$, 24H), 3.51 (m, N–CH_2–C_6H_{13}, 8H), 3.54 (s, C–CH_2–O, 8H), 3.70 (s, N–CH_2–CH_2–O–, 8H), 3.91 (s, N–CH_2–CH_2–O–, 8H). ^{13}C-NMR ([$^{2}H_6$]DMSO, ppm) 13.99 (–CH_3), 21.05, 22.94, 25.80, 28.29, 31.01(N– CH_2–$(CH_2)_5$–CH_3), 44.44 (quaternary C), 50. 82 (–N $(CH_3)_2$), 62.69(N–CH_2–C_6H_{13}), 64.44, 64.55 (O–CH_2– CH_2–N), 70.00 (C–CH_2–O). IR (KBr): 3483, 2927, 2859, 1467, 1118 cm^{-1}.

The synthesized displacers were also analyzed by elemental analysis and were found to be within acceptable ranges.

3.3.3. Displacer analysis

Whenever possible, UV–vis absorption was used to monitor the column effluent during linear gradient analysis (e.g. benzylamine, aniline, 1,4,8,11-tetrazacyclotetradecane, PE-DMABzCl4, PhTMA6 and DPE-DMABzCl6). For non-UV absorbing displacers, fractions of the column effluent were collected and analyzed for the displacer as follows:

Primary amine-containing displacers (e.g. spermine, spermidine, butylamine, methylamine, tetraethylenepentamine, triethylenetetramine, diethylenetriamine, bis(hexamethylene)triamine, pentaerythrityltetramine and neomycin) were determined by complexation with fluorescamine [37,38]. The fractions containing the displacer were diluted such that the resulting concentration of amine moieties in the sample was between 0.01 and 0.1 m*M*. A 0.28 mg/ml fluorescamine solution in acetone was then added to the sample in 1:3 (v/v) ratio. Excitation at 390 nm and emission at 475 nm were used to quantitate the amount of displacer in the sample.

Quaternary ammonium-containing displacers (e.g. PETMA4, DPE-TMA6, PE-DMAHepI4, PE-DMACyI4) were determined by complexation with bromophenol blue followed by extraction of the complex [39]. The displacer fractions were diluted to a concentration range of 0.5–2 m*M*. To 1 ml of the sample were added 0.1 ml of 10% (w/v) Na_2CO_3 and 1 ml of 0.4 mg/ml bromophenol blue (made in 0.01 *M* NaOH). The contents of the tube were mixed to ensure a complete reaction. The complex was then extracted by 2 ml of chloroform over a period of 1–2 h. The absorbance of the aqueous layer was recorded at 590 nm with the absorbance being inversely related to the concentration of the quaternary ammonium compound in the sample.

4. Results and discussion

In order to study the effects of various structural features on the efficacy of low-molecular-mass displacers, several cationic molecules were arranged in homologous series. The molecules in each series differed from each other in predominantly one structural characteristic. This enabled us to sys-

tematically probe the key factors which determine the affinity of low-molecular-mass displacers.

This study was carried out on three different stationary phases: a polymethacrylate (PMA)-based stationary phase (Waters SP-8HR), an agarose-based material (Pharmacia high-performance SP Sepharose) and a hydrophilized PS–DVB support (Poros HS50). These phases are representative of different classes of stationary phases typically employed for protein chromatography.

Agarose is an alternating copolymer of (1-3)-β-D-galactopyranose and (1-4)-3,6-anhydro-α-L-galactopyranose and is known to be very hydrophilic due to the presence of a high density of free OH groups [40]. The functional group (propane sultone) is added at the free hydroxyl sites, either directly or through spacer groups such as epichlorohydrin and 1,4 butanediol diglycidyl ether [41]. The long alkyl chains of the spacer groups may impart some hydrophobic character to this resin. The Waters SP-8HR has a backbone of poly(methyl methacrylate) crosslinked with ethanedimethacrylate [40]. The spacer groups and the functionalization are similar to those on the agarose based resin. Copolymers of styrene and divinylbenzene (PS–DVB) are also widely used for commercial ion exchangers [41]. Since the hydrophobic nature of these resins makes them unsuitable for protein chromatography, several schemes have been designed to make these resins more hydrophilic [42,43]. The Poros stationary phases (Perkin-Elmer) and the Source series of ion exchangers (Pharmacia) are two of the commercial hydrophilized PS–DVB resins used for protein chromatography. One of the successful schemes to render the stationary phase more hydrophilic has involved the adsorption of polymers containing hydrophilic and hydrophobic segments onto the polymeric support followed by cross-linking to produce a permanent hydrophilic film [24]. One of the techniques involves the epoxy monomers, epichlorohydrin and glycidol which are polymerized with boron trifluoride to give a polymer with alternating hydrophobic and hydrophilic segments. When this polymer is adsorbed onto the PS–DVB beads, the hydrophobic $-CH_2Cl$ groups orient towards the surface while the $-CH_2OH$ groups orient towards the solution where they can be functionalized. The polymer is then cross-linked to the PS–DVB support by ether bond formation in strong base [24]. Clearly, these different classes of ion exchangers can be expected to have different non-specific (e.g. hydrophobic, hydrogen bonding) interactions with displacers.

Fig. 2 shows the relative ranking of four molecules containing a single primary amine functionality. The ranking order on all three supports followed the order benzylamine>butylamine>methylamine. While there existed a clear difference between the affinities of butylamine and methylamine on the Waters SP-8HR (Fig. 2a) and Poros HS50 (Fig. 2c) supports, there was essentially no difference in their affinities on the Sepharose material (Fig. 2b). These results indicate that hydrophobic interactions can play a more significant role in the affinity of solutes in the Waters and Poros materials as compared to the Sepharose material.

Fig. 3 shows a comparison between three different geometries — linear, branched and cyclic; with all three molecules possessing the same number of charges. The ranking order on the Waters SP-8HR (Fig. 3a) and SP Sepharose (Fig. 3b) stationary phases were: linear>branched>cyclic. However, the order between the branched and linear structures was reversed on the Poros HS50 stationary phase (Fig. 3c), with the branched molecule being superior to the linear one. The differences in the affinities of the different geometries may be related to the spacing of the charges on various stationary phase materials.

Fig. 4 shows the ranking of various linear displacer molecules on the three stationary phase materials. The comparisons demonstrate the effect of increasing the number of charges on the linear molecules, Fig. 4a–c show a comparison of spermine (4 charges) to spermidine (3 charges); while Fig. 4d–f compare molecules with 5, 4 and 3 charges respectively (note: the spacing between the charges is the same in each of these two sets of molecules). The ranking order on all three stationary phases indicates that efficacy increases with an increase in the number of charges on the molecules.

Fig. 5 shows the effect of the spacing between the charges for linear displacers. For spermine and triethylene tetramine, the greater the spacing between charges, the higher the affinity on all three stationary phases (Fig. 5a, c and e). A similar trend is observed in the case of spermidine and diethylene triamine which both have three charges (Fig. 5b, d and f).

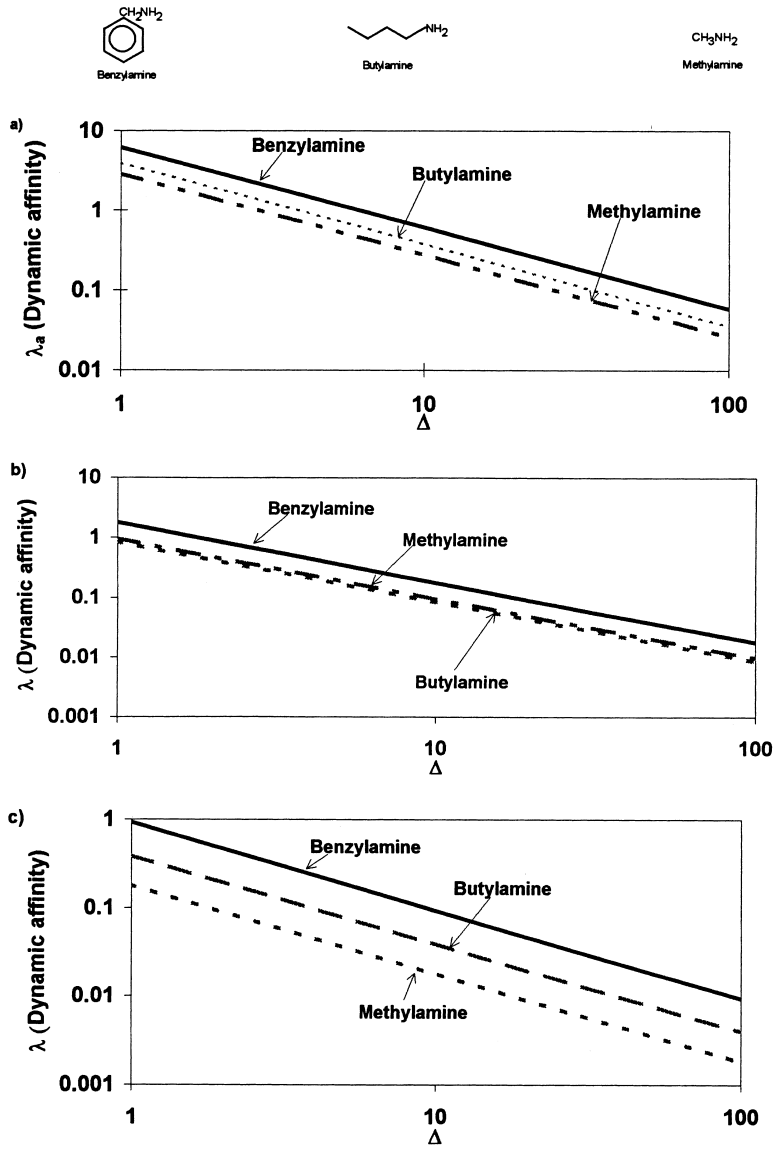


Fig. 2. Ranking of singly charged amines (a) on Waters SP-8HR (polymethacrylate based); parameters: benzylamine ($\nu=1$, $K=10.1$), butylamine ($\nu=1$, $K=7.84$), methylamine ($\nu=1$, $K=5.84$) (b) on SP-Sepharose (agarose based); parameters: benzylamine ($\nu=1$, $K=1.79$), butylamine ($\nu=1$, $K=0.86$), methylamine ($\nu=1$, $K=0.96$) (c) on Poros HS50 (polystyrene–divinylbenzene with hydrophilic coating); parameters: benzylamine ($\nu=1$, $K=0.94$), butylamine ($\nu=1$, $K=0.39$), methylamine ($\nu=1$, $K=0.18$).

However, this trend does not appear to hold when there is a further increase in the spacing between the charges. For example, bis(hexamethylene) triamine has a greater spacing between its charges than spermidine, however its affinity is lower than that of spermidine. Interestingly, while the three resins have the same qualitative trends the relative affinities are different on the various materials. While the affinity of the bis (hexamethylene) triamine is close to that of spermidine on the Waters material (Fig. 5b) it is closer to that of diethylene triamine on the Poros material (Fig. 5f). Nevertheless, these results indicate

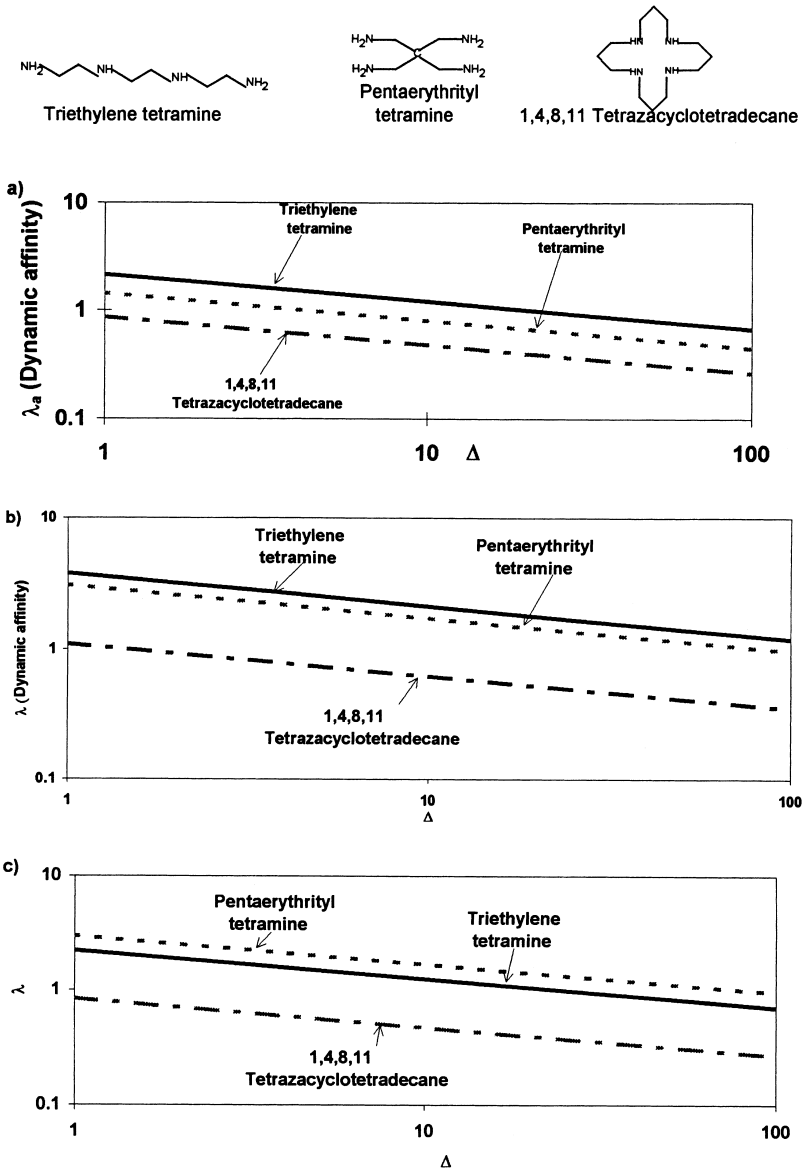


Fig. 3. Ranking of linear, branched and cyclic geometries (a) on Waters SP-8HR; parameters: triethylenetriamine ($\nu=4$, $K=20.0$), pentaerythrityltetramine ($\nu=4$, $K=4.12$), 1,4,8,11-tetrazacyclotetradecane ($\nu=4$, $K=0.58$) (b) on SP-Sepharose; parameters: triethylenetriamine ($\nu=4$, $K=87.7$), pentaerythrityltetramine ($\nu=4$, $K=203.1$), 1,4,8,11-tetrazacyclotetradecane ($\nu=4$, $K=1.43$) (c) on Poros HS50; parameters: triethylenetriamine ($\nu=4$, $K=24.5$), pentaerythrityltetramine ($\nu=4$, $K=79.7$), 1,4,8,11-tetrazacyclotetradecane ($\nu=4$, $K=0.51$).

that there is a limit to the affinity that can be attained by increasing charge spacing in linear displacers.

Fig. 6 shows the relative ranking of branched displacer molecules (PETMA4, DPE-TMA6, PhTMA6, PE-DMABzCl4 and DPE-DMABzCl6) synthesized in our laboratory. The addition of aromatic functionalities on the outer surface of the molecules (e.g. PE-DMABzCl4 and DPE-DMABzCl6) results in a dramatic increase in the affinity on both the Waters SP-8HR (Fig. 6a) and

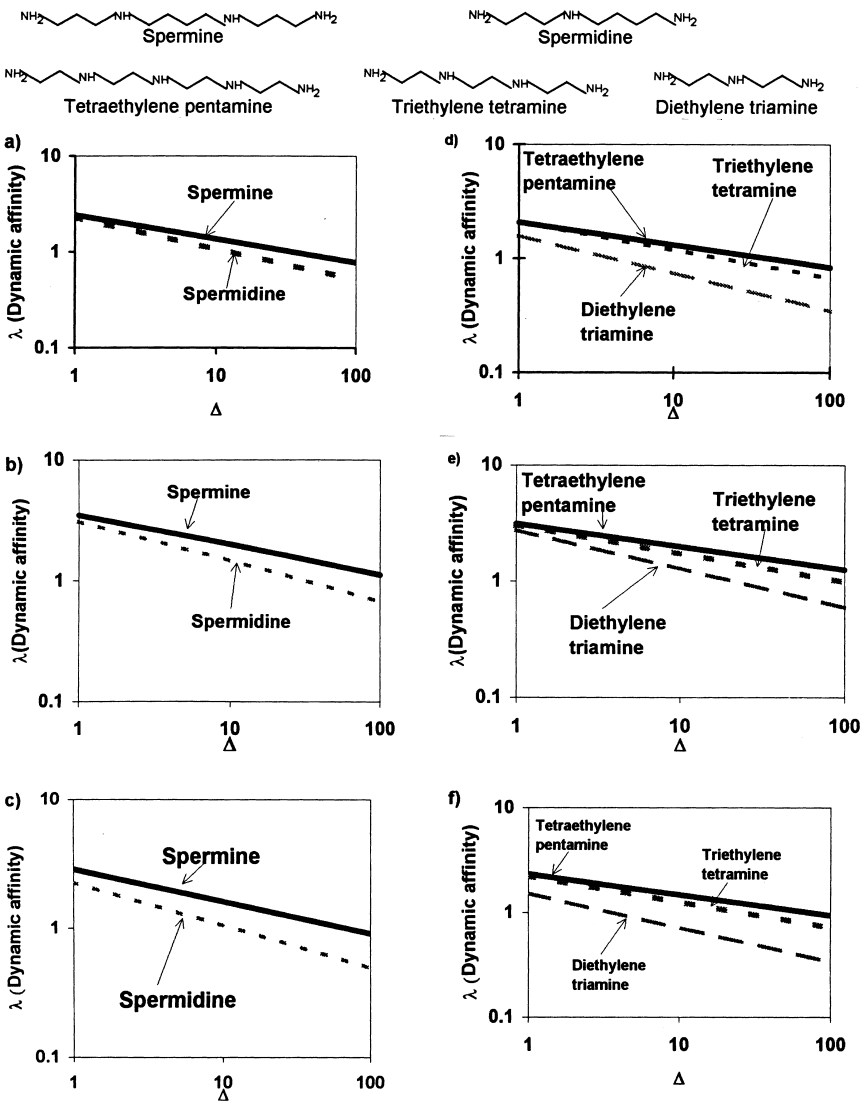


Fig. 4. Ranking of linear amines, effect of increasing the number of charges (a) and (d) on Waters SP-8HR; parameters: spermine ($\nu=4$, $K=36.3$), spermidine ($\nu=3$, $K=12.6$), tetraethylenepentamine ($\nu=5$, $K=40.3$), triethylenetetramine ($\nu=4$, $K=20.0$), diethylenetriamine ($\nu=3$, $K=4.0$) (b) and (e) on SP-Sepharose; parameters: spermine ($\nu=4$, $K=151.5$), spermidine ($\nu=3$, $K=30.44$), tetraethylenepentamine ($\nu=5$, $K=301.19$), triethylenetetramine ($\nu=4$, $K=87.7$), diethylenetriamine ($\nu=3$, $K=20.74$) (c) and f on Poros HS50; parameters: spermine ($\nu=4$, $K=67.9$), spermidine ($\nu=3$, $K=11.75$), tetraethylenepentamine ($\nu=5$, $K=68.18$), triethylenetetramine ($\nu=4$, $K=24.54$), diethylenetriamine ($\nu=3$, $K=3.56$)

Poros HS50 (Fig. 6c) stationary phase materials. However, there is a more significant difference in the affinities of PE-DMABzCl4 and DPE-DMABzCl6 on the Poros material. The affinity of PhTMA6, which has the aromatic unit as its core, is intermediate between those of the aromatic displacers (PE-DMABzCl4 and DPE-DMABzCl6) and the non-aromatic molecules (PETMA4 and DPE-TMA6) on these two phases. These results indicate that the addition of aromatic moieties, particularly on the outer surface of the molecule, can result in a significant increase in the affinity of displacers on the

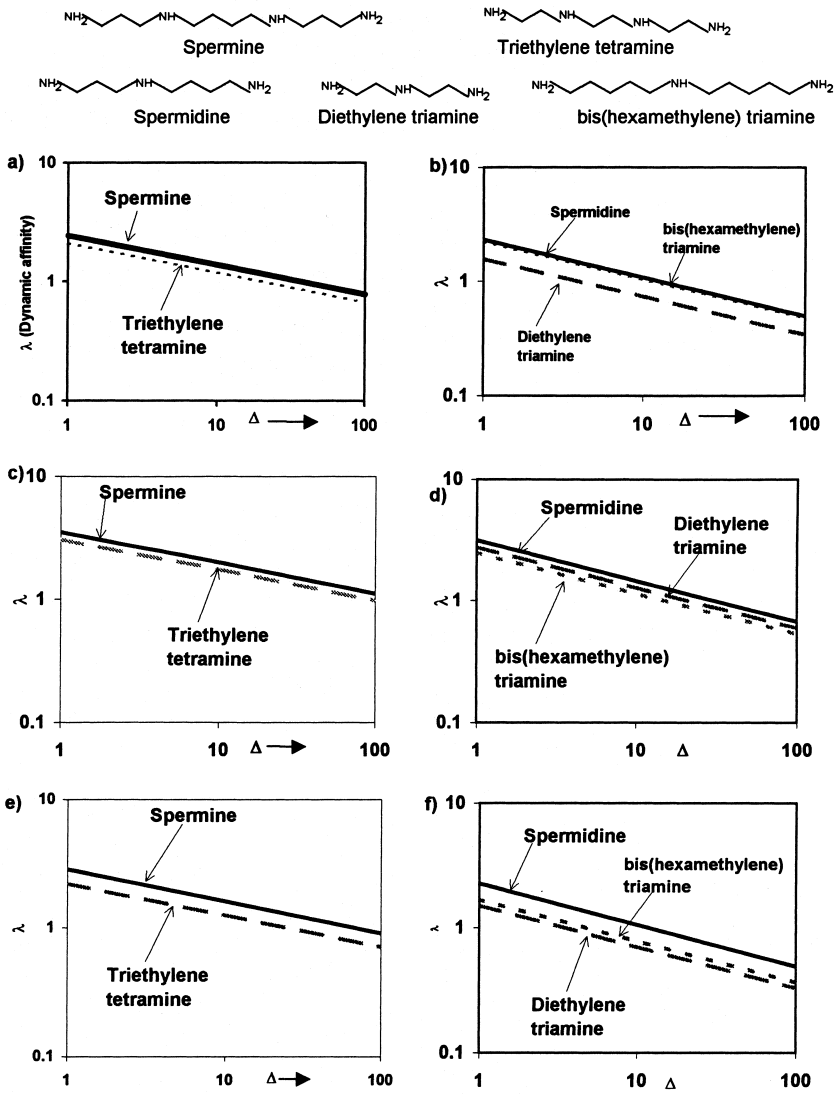


Fig. 5. Ranking of linear amines, effect of charge spacing. Parameters as in Fig. 4, bis(hexamethylene)triamine (b) on Waters SP-8HR ($\nu=3$, $K=11.02$) (d) on SP Sepharose ($\nu=3$, $K=14.9$) (f) on Poros HS50 ($\nu=3$, $K=4.85$).

PMA and PS–DVB based stationary phases. It is believed that this trend is due in part to the greater hydrophobicity of these stationary phases.

In contrast, the effect of adding aromatic groups on displacers is less pronounced on the agarose based SP Sepharose stationary phase. This trend can be seen in Fig. 6b, where the affinity lines of the displacers are closer together than on the other phases. These results indicate that this carbohydrate based stationary phase is less responsive to hydrophobic contributions. The results in this series (Fig. 6a–c) indicate that there can be substantial interactions between low-molecular-mass displacers and the backbone structure on chromatographic stationary phases.

In order to study the hydrophobic contributions of displacer affinity, a homologous series of molecules was synthesized as described in the experimental

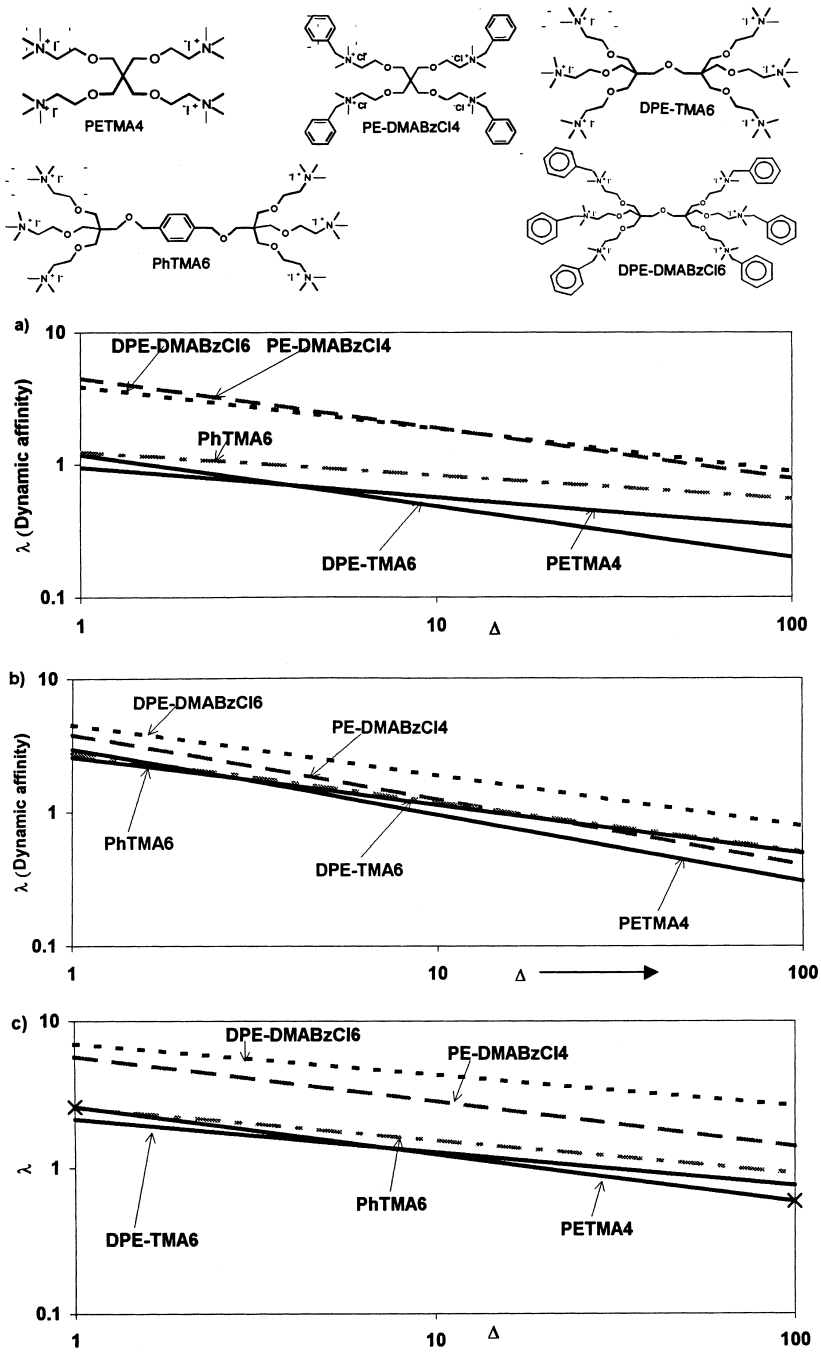


Fig. 6. Ranking of branched displacers. Parameters: (a) on Waters SP-8HR: PETMA4 ($\nu=2.6$, $K=1.52$), DPE-TMA6 ($\nu=4.47$, $K=0.79$), PhTMA6 ($\nu=5.65$, $K=3.47$), PE-DMABzCl4 ($\nu=3.12$, $K=70.2$), DPE- DMABzCl6 ($\nu=3.14$, $K=72.1$) (b) on SP-Sepharose: PETMA4 ($\nu=2.03$, $K=9$), DPE-TMA6 ($\nu=2.79$, $K=13.92$), PhTMA6 ($\nu=2.71$, $K=15.63$), PE-DMABzCl4 ($\nu=2.05$, $K=15.58$), DPE-DMABzCl6 ($\nu=2.64$, $K=53.38$) (c) on Poros HS50: PETMA4 ($\nu=3.12$, $K=18.97$), DPE-TMA6 ($\nu=4.45$, $K=28.56$), PhTMA6 ($\nu=4.5$, $K=67.38$), PE-DMABzCl4 ($\nu=3.25$, $K=287.18$), DPE-DMABzCl6 ($\nu=4.7$, $K=9133.6$).

section. This series, shown in Fig. 7, was employed to examine whether the enhancement in affinity for branched molecules with benzyl units at their termini is due to the hydrophobic effect alone or whether it involves some specific aromatic interactions. The molecules in this series are based on the PETMA4 structure with the R group on the quaternary ammonium group consisting of either a benzyl (PE-DMABzCl4), a cyclohexyl (PE-DMACyI4), a heptyl (PE-DMAHepI4) or a methyl unit (PETMA4). The cyclohexyl group has comparable hydrophobicity as the benzyl functionality, but lacks aromaticity. As seen in Fig. 7 there is a dramatic difference in the relative affinities of these homologous displacers on the three stationary phases. The trends on the Waters SP-8HR material (Fig. 7a) indicate an affinity ranking of PE-DMABzCl4>PE-DMAHepI4>PE-DMACyI4>PETMA4. This trend indicates that all of the molecules with hydrophobic R groups have higher affinity than the PETMA4. In addition, the results indicate that aromaticity plays an important role in the affinity of these molecules (e.g. PE-DMABzCl4>PE-DMACyI4).

The trends on the Poros HS50 resin (Fig. 7c) shows an affinity ranking of PE-DMAHepI4>PE-DMABzCl4=PE-DMACyI4>PETMA4. Again, the incorporation of hydrophobic R groups results in an elevated affinity. In contrast to the results on the Waters material, however, the PE-DMABzCl4 had comparable affinity to PE-DMACyI4. Thus, aromaticity does not appear to play as important a role on this material.

In sharp contrast to the results on the Waters and the Poros materials, there is minimal difference in the affinities of these homologous displacers on the SP-Sepharose stationary phase (Fig. 7b). These results are quite compelling in that they clearly demonstrate the marked difference in the affinity of different displacers on various stationary phase materials. Further, the homologous nature of this series of molecules eliminates any ambiguity in the results.

In order to compare the relative efficacy of the various classes of displacers, the highest affinity linear and branched displacers for each stationary phase are plotted on Fig. 8. In addition, the displacer neomycin [12] is included in these figures. As seen in the results, the highest affinity displacers for the Waters (Fig. 8a) and the Poros (Fig. 8c) materials were the branched structures. On the PMA based Waters SP-8HR stationary phase, the displacers with aromatic functionalities on the outer surface (e.g. DPE-DMABzCl6) possessed the highest affinity, while on the Poros material, PE-DMAHepI4, with heptyl functionalities on its outer surface possessed the highest affinity. The relative affinities of spermine (the highest affinity linear displacer) and Neomycin were markedly different on the three phases. On the Waters material (Fig. 8a) spermine had a higher affinity than neomycin, although the affinities were close. On the Poros material (Fig. 8c), neomycin had a higher affinity than spermine, although both displacers had a significantly lower affinity than the branched structures. In contrast to these results, on the Sepharose material (Fig. 8b), neomycin had a higher affinity than the both spermine and the branched displacers. These results again confirm that the lack of hydrophobic interactions in the Sepharose material results in a dramatic difference in the relative affinities of these different classes of displacers.

5. Conclusions

The results presented in this manuscript indicate that high affinity, low-molecular-mass displacers can indeed be designed for different stationary phase materials. Furthermore, the data indicate that these displacers will require structural characteristics that will differ depending on the chemistry of the stationary phase material. While aromaticity played an important role on the methacrylate-based material, hydrophobicity was more significant on the hydrophilized polystyrene–divinyl benzene material. On the other hand, for agarose based materials, aromaticity and/or hydrophobic interactions did not appear to play a major role. These results are in agreement with the nature of the stationary phase backbone; agarose is a hydrophilic material while both the PMA and the coated PS–DVB supports are more hydrophobic. Clearly, the choice of low-molecular-mass displacers for a given stationary phase must include a consideration of these non-specific interactions. This study provides guidelines for the design of high affinity, low-molecular-mass displacers on different stationary phases and lays the

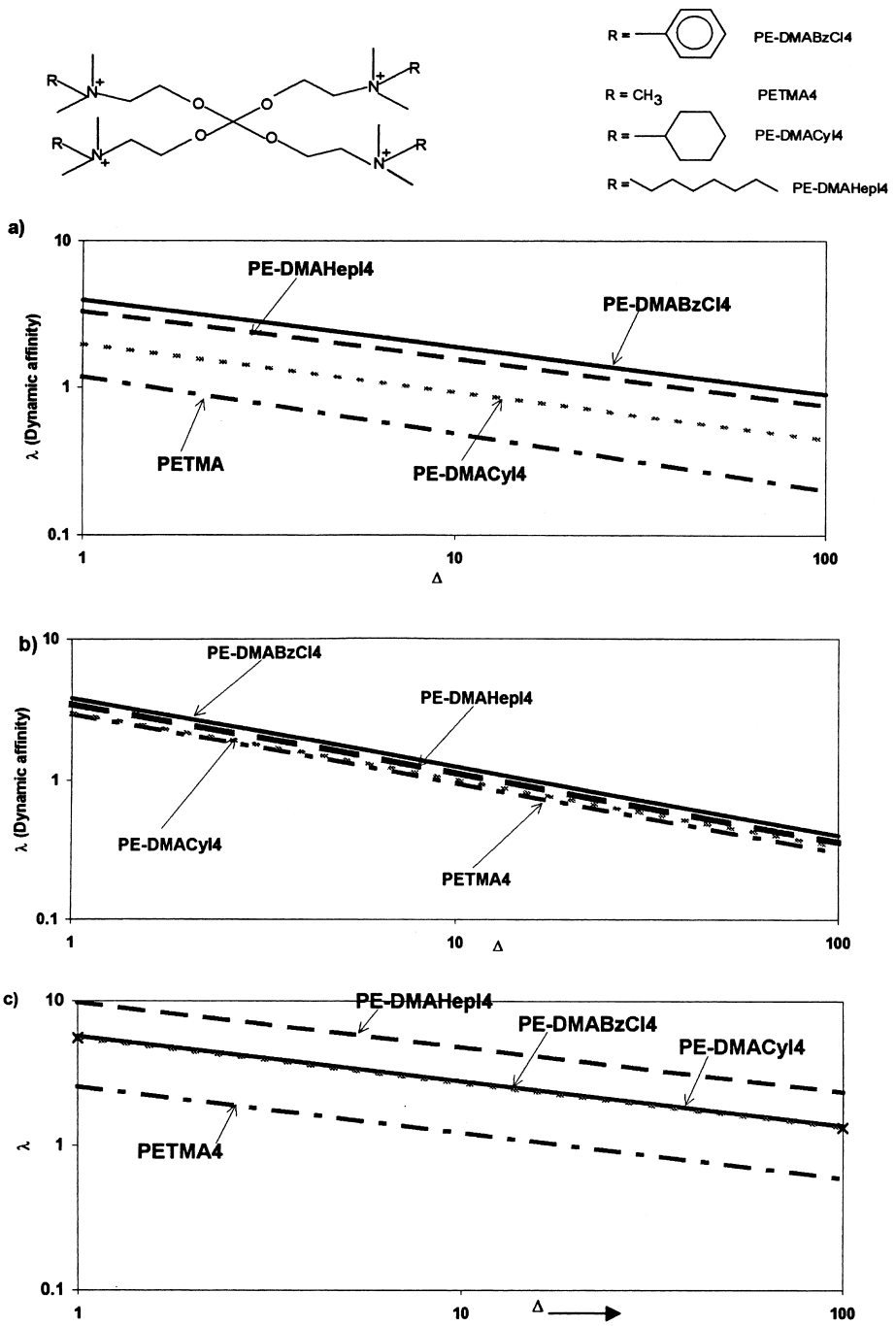


Fig. 7. Ranking of branched displacers based on PETMA4 — effect of hydrophobicity/aromaticity. Parameters: (a) on Waters SP-8HR: PE-DMABzCl4 ($\nu=3.12$, $K=70.2$), PE-DMAHepI4 ($\nu=3.12$, $K=40.0$), PE-DMACyI4 ($\nu=3.1$, $K=7.9$), PETMA4 ($\nu=2.6$, $K=1.5$); (b) on SP Sepharose: PE-DMABzCl4 ($\nu=2.05$, $K=15.6$), PE-DMAHepI4 ($\nu=2.03$, $K=12.5$), PE-DMACyI4 ($\nu=2.1$, $K=10.1$), PETMA4 ($\nu=2.03$, $K=9.0$); (c) on Poros HS50: PE-DMABzCl4 ($\nu=3.3$, $K=287.2$), PE-DMAHepI4 ($\nu=3.20$, $K=1631.5$), PE-DMACyI4 ($\nu=3.25$, $K=258.7$), PETMA4 ($\nu=3.12$, $K=19$).

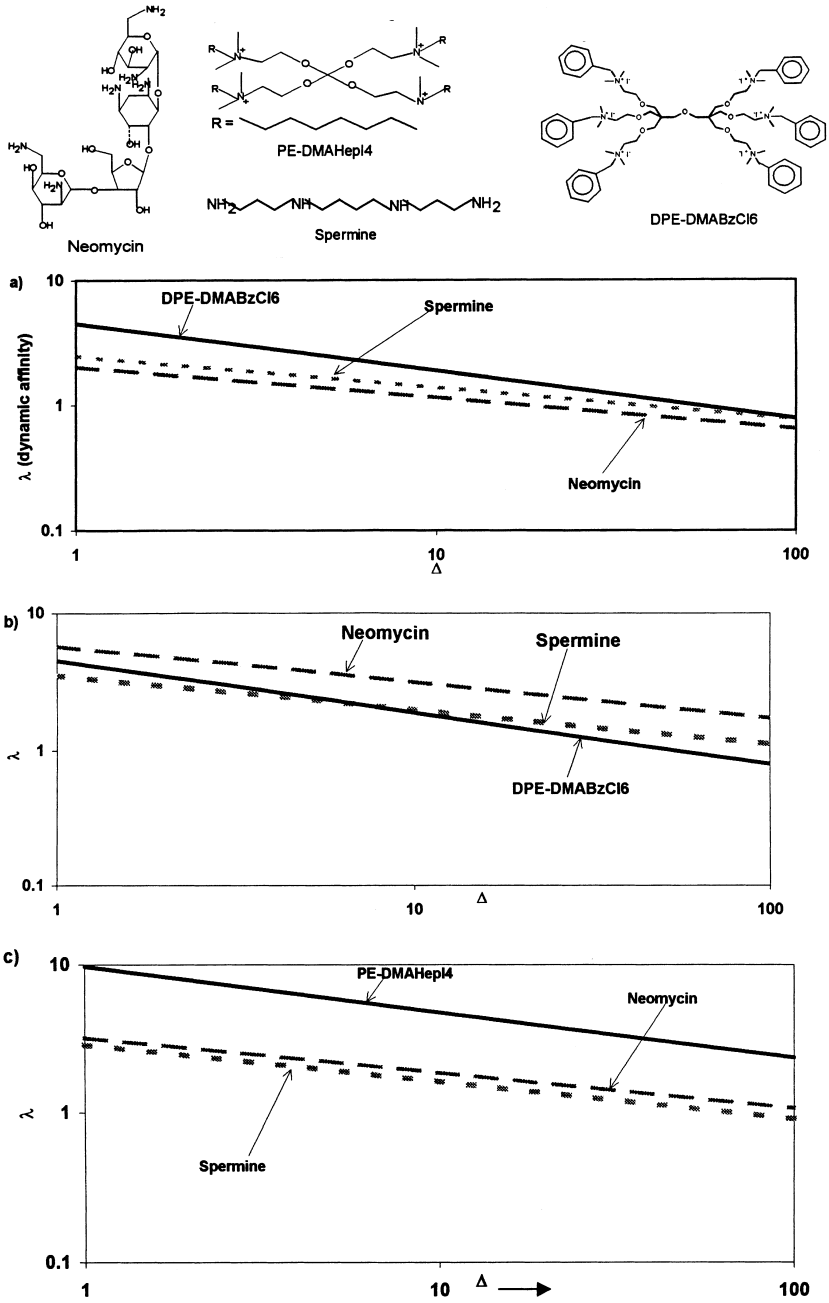


Fig. 8. Ranking of neomycin, the best linear and branched displacers on (a) Waters SP-8HR:, Neomycin ($\nu=4.07$, $K=17.9$), DPE-DMABzCl6 ($\nu=3.14$, $K=72.1$), spermine ($\nu=4$, $K=36.3$) (b) SP Sepharose: Neomycin ($\nu=3.83$, $K=798.5$), DPE-DMABzCl6 ($\nu=2.64$, $K=53.38$), spermine ($\nu=4$, $K=151.5$) (c) Poros HS50: Neomycin ($\nu=4.2$, $K=135.2$), PE-DMAHepI4 ($\nu=3.20$, $K=1631.5$), spermine ($\nu=4$, $K=67.9$).

foundation for more detailed investigations into the interactions of small molecules with chromatographic stationary phases.

Acknowledgements

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