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CHEMICALLY SYNTHESIZED HYDROPHOBIC ANION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY SUPPORTS USED FOR OLIGONUCLEOTIDE RESOLUTION BY MIXED MODE CHROMATOGRAPHY

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SUMMARY

A commercially available aminopropylsilyl bonded-phase high-performance liquid chromatography support has been modified with three different organic acids each containing an amine group and a hydrophobic moiety to produce anion-exchange supports with increased hydrophobicity. The chromatographic characteristics of these supports were examined using two oligonucleotides of equal chain length but differing hydrophobic character, $(Ap)_3A$ and $(Up)_3U$. The resolution of these two solutes on the modified supports in the pH range 4.5–6.5 and an acetonitrile concentration of 0–48% is described. The most effective support contains a phenylalanine moiety (APS-PHE) bound through an amide to the commercial support. This support has been used to resolve oligonucleotides of the same size (6–12 residues) but different sequence prepared enzymatically or chemically. The difficulties inherent in parameter optimization in mixed-mode chromatography are discussed.

INTRODUCTION

Since oligonucleotides are essentially polymeric anions (polyphosphates) containing hydrophobic moieties (nucleobases), both anion-exchange and reversed-phase chromatography are useful for the resolution and isolation of a particular species. Chromatographic supports which employ both types of interaction, such as benzoylated[1] or naphthoylated[1] DEAE-cellulose, the RPC-5 material[2], and more recently ODS-Hypersil containing a dynamically bound tetraalkylammonium salt[3], are of particular value for the isolation of oligonucleotides or nucleic acids.

DEAE-Cellulose and DEAE-Sephadex are very commonly used for oligonucleotide separations. Both supports have been generally considered to function as pure anion exchangers, but the separation on DEAE-cellulose of oligomers of the same chain length but different purine pyrimidine content has been described[4]. Further studies have indicated that both hydrophobic and hydrogen bonding interactions are involved in this chromatographic behaviour[5,6].

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More recently, bonded phase microparticulate silica-based chromatographic supports designed for use with high-performance liquid chromatography (HPLC) systems have significantly increased chromatographic resolution. Reversed-phase HPLC supports are generally more popular for oligonucleotide analysis since it is reasonably easy to optimize chromatographic parameters[7]. Anion-exchange chromatography on HPLC supports is also useful for resolution of oligonucleotides. However, the parameters governing anion-exchange chromatography are more difficult to optimize since mixed-mode chromatography appears to occur even on supports thought to be purely anion exchangers[8]. The present HPLC supports have been designed such that both the ionic and the hydrophobic interactions can be altered in order to optimize separations.

EXPERIMENTAL

Materials

APS-Hypersil (5 μm) was purchased from Shandon (Runcorn, U.K.). $(Up)_3U$ and $(Ap)_3A$ were from P.L. Biochemicals (St. Goar, F.R.G.). 2-Pyridylacetic acid hydrochloride and *p*-(diethylamino)-benzoic acid hydrochloride were purchased from Aldrich Europe and from Aldrich (Milwaukee, U.S.A.), respectively. L-α-Phenylalanine was from Sigma (Munich, F.R.G.). RNAse T_1 was from Sankyo through Koch-Light (Frankfurt, F.R.G.). $tRNA^{Phe}$–C–C–A from baker's yeast has been prepared according to ref. 9. Acetonitrile was of HPLC grade from Baker (Deventer, The Netherlands). All other reagents were of analytical grade.

Methods

HPLC was performed on a Du Pont 850 gradient liquid chromatograph consisting of a pump, microprocessor, variable-wavelength detector and thermostatically controlled column compartment set at 35°C.

Support modification method A. To 8 mmol (1.39 g) of 2-pyridylacetic acid hydrochloride and 8 mmol (1.11 g) of *p*-nitrophenol in 32 ml of dry dioxane and 6 ml of dry pyridine was added a solution of 8 mmol (1.65 g) dicyclohexylcarbodiimide in 8 ml of dry dioxane. The reaction mixture stirred for 2 h at room temperature. The precipitated urea was filtered off and the filtrate was added directly to 4 g of dried APS-Hypersil (capacity 0.6 mmol amine/g). The suspension was shaken for 48 h at room temperature. The support was filtered using a glass frit and washed with *ca.* 50 ml each of the following solvents: dioxane–pyridine (1:1) (twice), methylene chloride (twice) and diethyl ether. After the last washing step the support was dried under vacuum at 45°C overnight. A small sample (100 mg) was submitted for elemental analysis.

Support modification method B. N-(*o*-Nitrophenylsulfenyl)-L-α-phenylalanine (NPS-Phe)[10] (8 mmol; 2.5 g) was dissolved in 130 ml of 50% dimethylformamide water and the pH was adjusted to 4.7 with 1 *N* hydrochloric acid. APS-Hypersil (2.5 g) was then added and the pH was re-adjusted to 4.7. To the suspension were added 16 mmol (3 g) of N-ethyl-N′-(3-dimethylaminopropyl)carbodiimide hydrochloride dissolved in 30 ml of water. The suspension was shaken for 48 h at room temperature. After the support had been filtered off it was washed and dried according to the

procedure described above. A sample of *ca.* 100 mg was submitted for elemental analysis.

Capping of the residual primary amine groups. To the dry modified APS-Hypersil suspended in 16 ml of methylene chloride containing 0.8 ml of dry pyridine were added 4 ml of acetic anhydride. The suspension was shaken for 1 h at room temperature. Afterwards the support was washed with methylene chloride (twice) and diethyl ether, and dried under vacuum at 45°C.

Removal of the o-nitrophenylsulfenyl blocking group. To 2.3 g of APS-Hypersil modified with NPS-Phe suspended in 100 ml of 0.2 *M* ammonium acetate, pH 5.1, containing 5% methanol were added 100 ml of 1.0 *M* $Na_2S_2O_3$. The suspension was shaken for 30 min until a pale yellow color was achieved. A sample of 100 mg was again submitted for an elemental analysis. Columns of 4.6 mm I.D. and lengths of 150 mm or 250 mm were packed as slurries in methanol at 400 bar as previously described[11].

RESULTS

Preparation of the hydrophobic anion-exchange supports

A commercially available aminopropylsilyl bonded phase support (APS-Hypersil) was chosen for a number of reasons. The primary amine provided a functional group that would be relatively easy to modify. Additionally this chromatographic support has two characteristics we wished to exploit: as a primary amine, its ability to act as an anion exchanger can be altered with pH; secondly, the propyl group should provide some of the desired hydrophobic interactions.

Using APS-Hypersil as a starting material we prepared three new chromatographic supports with various ionic and hydrophobic characteristics (Table I). We

TABLE I

HYDROPHOBIC ANION-EXCHANGE SUPPORTS

Support	*Structure*	*Percentage modification of APS-Hypersil**
APS	Hypersil $-CH_2-CH_2-CH_2-NH_2$	—
APS-PHE	Hypersil $-CH_2-CH_2-CH_2-NH-CO-CH(NH_2)-CH_2-C_6H_5$	45%
APS-DEA	Hypersil $-CH_2-CH_2-CH_2-NH-CO-C_6H_4-N(CH_2-CH_3)_2$	49%
APS-PYR	Hypersil $-CH_2-CH_2-CH_2-NH-CO-CH_2-C_5H_4N$	58%

* Calculated from nitrogen analysis.

employed two coupling techniques, one in anhydrous solution and one in aqueous solution. In the first the *p*-nitrophenyl ester of the desired acid was prepared and the coupling was done in dry organic solvent. In the second a peptide-type coupling was done using a water-soluble carbodiimide. With both methods *ca.* 50% of the starting primary amine groups could be modified as measured by elemental analysis. The residual amino groups were "capped" using acetic anhydride to prevent them from influencing ionic interactions during chromatography. A negative ninhydrin test confirmed the absence of primary amines. In the case of the APS-PHE column the amine group of the amino acid remained protected as an *o*-nitrophenylsulphenimide until the capping reaction was complete. Deprotection was as described (see Experimental section). Determination of the coupling yield was based on elemental analysis for nitrogen. (Typical values: APS, 0.83%; APS-PHE, 1.11%; APS-DEA, 1.16%; APS-PYR, 1.18%.)

pH effects

$(Ap)_3A$ and $(Up)_3U$ were chromatographed isocratically in 0.3 *M* potassium phosphate at three different pH values using the four anion-exchange supports. The capacity factors (k') ($k' = V_r - V_0/V_0$, where V_r = solute retention volume and V_0 = column void volume) for this experiment are listed in Table II. The more hydrophobic $(Ap)_3A$ exhibits higher k' values on all columns at all three pH values than does $(Up)_3U$. The k' values are generally highest for the APS-PHE support and lowest for the APS-PYR support, with the APS-DEA and APS supports intermediate. The more basic primary aliphatic amines of the APS-PHE and APS supports exhibit a more pronounced pH dependence than the tertiary amines of the APS-DEA and APS-PYR supports. There is however a slight crossover effect which can be best observed from the values of $(Ap)_3A$ illustrated in Fig. 1.

TABLE II

k' VALUES FOR $(Up)_3U$ AND $(Ap)_3A$

	pH	*Chromatographic support*			
		APS	*APS-PHE*	*APS-DEA*	*APS-PYR*
$(Up)_3U$	4.5	6.0	5.5	5.0	7.0
	5.5	4.0	3.5	4.0	4.2
	6.5	3.5	2.8	3.0	4.0
$(Ap)_3A$	4.5	34.8	58.3	34.0	29.2
	5.5	14.8	27.2	20.5	13.2
	6.5	7.2	8.8	12.5	8.5

Organic modifier effects

By varying the acetonitrile concentration in the mobile phase from 0 to 48% the chromatographic characteristics of $(Ap)_3A$ and $(Up)_3U$ have been analyzed at pH 5.1 on two chromatographic supports. APS-PHE was chosen for comparison with the APS support since it exhibited generally the highest k' values of the present modified supports. Since an organic modifier in reversed-phase chromatography is

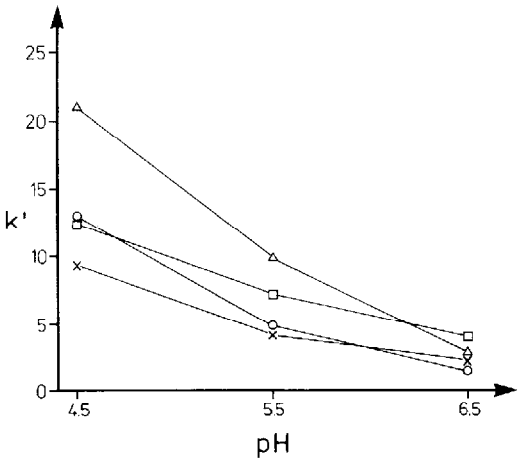

Fig. 1. Dependence of the k' value on pH using the elution of $(Ap)_3A$ from: (○) APS, (△) APS-PHE, (□) APS-DEA and (+) APS-PYR isocratically in a mobile phase of 0.3 *M* potassium phosphate at pH 4.5, 5.5 and 6.5.

used to alter the hydrophobic interactions between the solute and the support, we expected that different concentrations of acetonitrile would significantly affect the k' values for the two oligonucleotides. Additionally at high acetonitrile concentrations the chromatography should be the result of purely ionic interactions, and the two oligomers should elute together, being of equal chain length.

Fig. 2 clearly shows that for both supports a higher k' value is observed for $(Ap)_3A$ as expected, since adenosine is more hydrophobic than uridine. Additionally the higher reversed-phase character of the APS-PHE column in comparison with the APS column results in higher k' values on the APS-PHE column for $(Ap)_3A$ (k' = 86) and $(Up)_3U$ (k' = 5.8) than on the APS column (k' = 23 and 2.3, respectively). It is noteworthy that at the highest concentration of acetonitrile used (48%) the two oligomers could not be eluted together from either support.

The resolution of two solutes is defined as: $R_{21} = 2(R_2 - R_1)/W_2 + W_1$ where R_{21} = resolution of peak 2 with respect to peak 1: R_2, R_1 = retention volume of peaks 2 and 1, respectively; W_2, W_1 = peak width at baseline for peaks 2 and 1, respectively. The resolution between the two tetramers has been calculated at various acetonitrile concentrations according to this equation. Whereas the resolution between $(Ap)_3A$ and $(Up)_3U$ is essentially independent of acetonitrile concentration on the APS support, the resolution on the modified support (APS-PHE) can be markedly increased by the addition of 10–20% of an organic modifier to the mobile phase (Fig. 3). The enhanced resolution is expressed as a better peak shape, particularly for the $(Ap)_3A$ solute. It can also be observed from Fig. 4 that the resolution of the two peaks on the APS-PHE support is higher at all concentrations of acetonitrile than that observed for the APS support.

Applications and parameter optimization

Clearly the optimization of chromatographic parameters for mixed-mode chromatography is more complex than for reversed-phase or ion-exchange chromatogra-

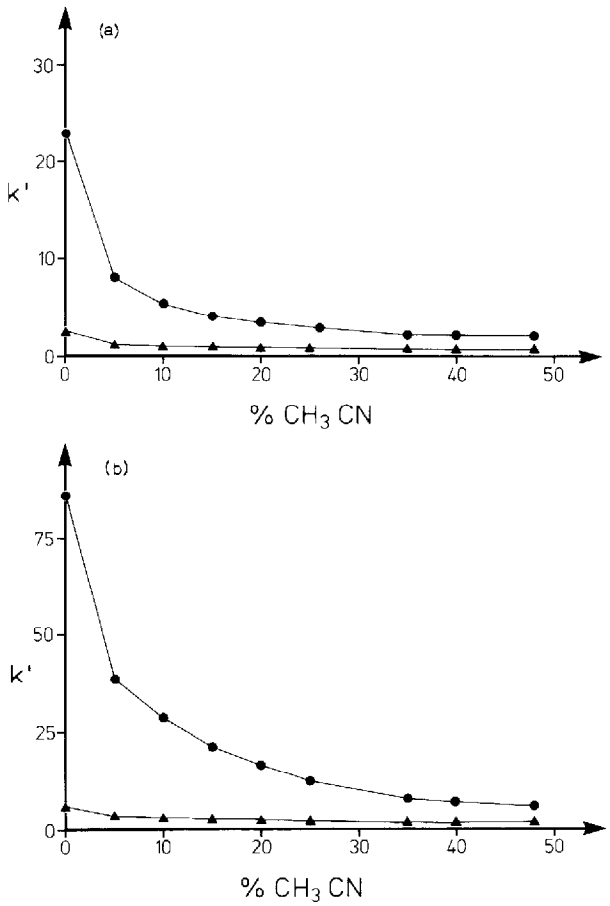

Fig. 2. Dependence of the k' value on the percentage of organic modifier (acetonitrile) in the mobile phase using the elution of (●) $(Ap)_3A$ and (▲) $(Up)_3U$ in 0.11 *M* potassium phosphate, pH 5.1, on APS support (a) and APS-PHE support (b).

phy. The mobile phase parameter that can be most easily altered to change the ratio of ion-exchange and reversed-phase interactions is the pH. Since the amine groups on these supports act as anion exchangers only when they are protonated, the number of sites available for ionic interaction is pH dependent. Therefore, as the pH of the mobile phase is increased and the number of protonated sites decreases, the ratio of hydrophobic sites to ionic sites will increase. Thus, at lower pH the support should have more ion-exchange character and at higher pH an increasing reversed-phase character.

The application of this kind of optimization is shown in Fig. 4. We have previously described the separation of oligoribonucleotides resulting from a ribonuclease T_1 digestion of $tRNA^{Phe}$ from yeast[11,12]. Three of the oligomers resulting from this digest include the hexamers ApApUpUpCpGp and ApUpUpUpApm^{2}Gp and the octamer m^{1}ApUpCpCpApCpApGp. A portion of the chromatogram containing these three oligonucleotides on the APS, APS-DEA and APS-PHE supports is shown

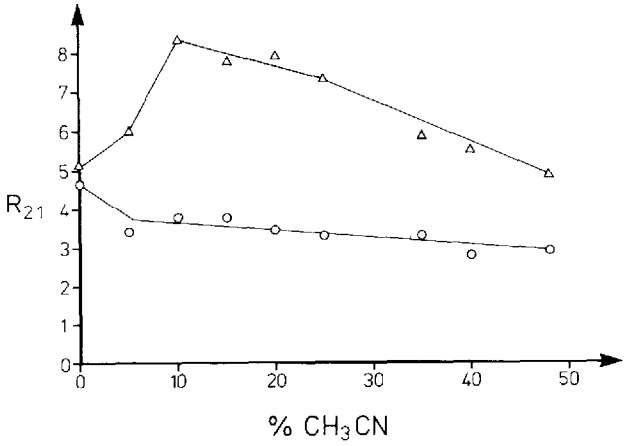

Fig. 3. Relation between the resolution (R_{21}) of $(Ap)_3A$ with respect to $(Up)_3U$, and the acetonitrile concentration for the two supports (○) APS and (△) APS-PHE using 0.11 *M* potassium phosphate isocratically at pH 5.1.

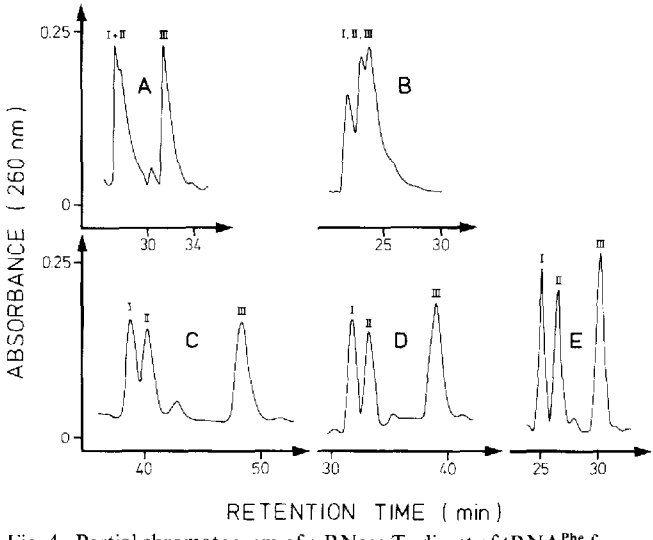

Fig. 4. Partial chromatogram of a RNase T_1 digest of $tRNA^{Phe}$ from yeast showing the resolution between the three oligonucleotides: (I) ApApUpUpCpGp, (II) ApUpUpUpApm^{2}Gp and (III) m^{1}ApUpCpCpApCpApGp using Buffer A (0.05 *M* potassium phosphate) and Buffer B (0.9 *M* potassium phosphate including 10% acetonitrile). Gradient, 0–60 min 100% Buffer A to 100% Buffer B. (A) APS support, pH 5.5; (B) APS-DEA support, pH 5.5; (C) APS-PHE support, pH 5.5; (D) APS-PHE support, pH 6.0; (E) APS-PHE support, pH 6.5.

in Figs. 4A, 4B and 4C, respectively. The commercial APS support is able to resolve the two hexamers from the octamer but resolution between the two hexamers is poor. The APS-DEA support, which has a higher hydrophobic character, is better able to resolve the two hexamers but less able to resolve the hexamers from the octamer. The APS-PHE column resolves the hexamers from the octamer and additionally

exhibits better resolution of the two hexamers. By repeating the chromatography of Fig. 4C (pH 5.5) at pH 6.0 (Fig. 4D) and pH 6.5 (Fig. 4E) two observations could be made. The retention times for all three oligomers decrease. This is expected since the ionic character of the support decreases but the mobile phase salt gradient remains the same. Secondly, as the pH increases, thus increasing the hydrophobic nature of the support, the resolution between the two hexamers increases such that in Fig. 4E essentially a baseline separation is achieved.

In some cases the APS-PHE support can resolve longer oligonucleotides of equal chain length which differ in sequence at only a single position. For example, the two complementary undecamers d(CpCpGpGpTpApApCpCpGpG) and d(CpCpGpGpTpTpApCpCpGpG) were prepared by solid-phase DNA synthesis on a silica support. For the fifth nucleotide coupling an equivalent mixture of the suitable adenosine and thymidine compound was used. After completion of the synthesis the two undecamers differing in sequence only at position 6 were deblocked and analyzed by HPLC. A commercial anion-exchange support containing a quaternary amine ($-NR_3^+$) was unable to resolve the two oligomers. At pH 6.5 either the APS or the APS-PHE support could discriminate the two oligomers. Higher resolution was however observed on the APS-PHE support. The HPLC analysis of the crude mixture on APS-PHE is shown in Fig. 5.

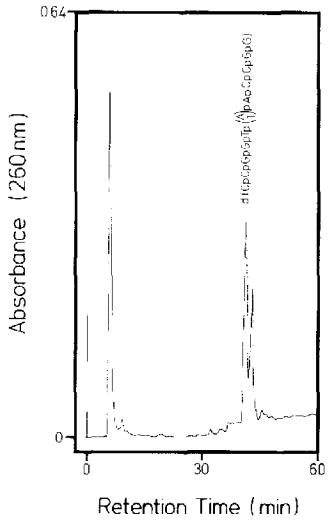

Fig. 5. Chromatogram of the crude mixture resulting from the chemical synthesis of two undecamers differing in sequence at position 6 (adenosine or thymidine) on the APS-PHE support using Buffer C (0.05 *M* potassium phosphate, pH 6.5, containing 10% acetonitrile) and Buffer D (0.9 *M* potassium phosphate, pH 6.5, containing 10% acetonitrile). Gradient, 0–60 min 100% Buffer C to 100% Buffer D.

DISCUSSION

Two different methods have been described to modify a commercially available aminopropylsilyl bonded phase silica (APS-Hypersil). Both methods result in *ca.* 50% modification of the primary amine groups present. Since, with respect to the aminopropyl moiety, a relatively bulky substituent has been covalently attached,

modification yields significantly above 50% seem unlikely. Acetic anhydride has been used to "cap" the remaining primary amine groups such that the chromatographic characteristics are largely a result of the covalent modification. The acylation with acetic anhydride was highly effective as measured by the ninhydrin test. It is possible that some primary amine groups were neither acylated nor accessible to the ninhydrin reagent and so remained on the surface of the support. However it is unlikely that these residual amine groups would significantly affect the chromatography of the relatively large oligonucleotides. On the other hand, the "capped" amine groups should increase the hydrophobic character of the support. This contribution to the hydrophobic interactions will become more significant with lower coupling yields and thus a higher percentage of "capped" amines.

The study involving pH effects clearly indicated that all four supports act as ion exchangers. The observation that $(Ap)_3A$ always exhibited a higher k' value than $(Up)_3U$ indicates the presence of hydrophobic interactions. The relation between the k' value and pH appears more pronounced for the APS and APS-PHE supports containing primary aliphatic amines (Fig. 1). This effect is probably related to the lower pK_B values for phenylalanine and *n*-propylamine (pK_B = 2–3) compared with those for diethylaniline and pyridine (pK_B = 8–9). Thus at a given pH the APS and APS-PHE supports have more ion-exchange character as a result of a higher degree of protonation than either the APS-DEA or APS-PYR supports. Conversely the latter two supports may tend to have a higher reversed-phase character at some pH values. This is indicated by the observation that $(Ap)_3A$ exhibits the highest k' value at pH 6.5 on the APS-DEA support in spite of the fact that both APS and APS-PHE, by virtue of their primary amines, will have a larger number of anion-exchange sites available.

Comparing the two supports with a common primary aliphatic amine one can observe that the addition of a hydrophobic phenyl group results in a large increase in k' for the hydrophobic oligomer $(Ap)_3A$. This is not as obvious in the case of $(Up)_3U$ since its hydrophobic character is reduced.

The hydrophobic interactions of both the APS and APS-PHE supports are clearly seen in Fig. 2. There is a much larger difference in k' values between $(Ap)_3A$ and $(Up)_3U$ for the APS-PHE support than observed for the APS support. The larger k' value for $(Ap)_3A$ on the APS-PHE column can clearly be attributed to hydrophobic or reversed-phase effects as a result of the covalently bound phenyl group. We were unable to elute the two tetramers together from either of the supports even with 48% acetonitrile in the mobile phase. Since these oligomers elute together from a purely reversed-phase column (ODS-Hypersil or Phenyl-Nucleosil) with this mobile phase, it is somewhat difficult to account for the present observations. It is possible that with a mixed-mode support and a high percentage of organic modifier in the mobile phase the initial interaction is purely ionic. However, as the oligomer is adsorbed onto the surface of the support by ionic interaction, a forced hydrophobic interaction may take place whereby some organic modifier is displaced from the surface of the support by the hydrophobic bases of the oligonucleotide.

Optimal resolution between $(Ap)_3A$ and $(Up)_3U$ on APS-PHE occurs with *ca.* 10% acetonitrile in the mobile phase (Fig. 3). This again appears to reflect the hydrophobicity differences between the two supports since no corresponding effect was observed on the APS support. The peak shape observed for $(Ap)_3A$ on the APS-

PHE support is significantly improved with a small amount of organic modifier. This would suggest that with respect to oligonucleotides, the hydrophobic character of this support is quite high and that some organic modifier is necessary to assist efficient migration along the surface of the support.

Parameter optimization in mixed-mode chromatography will remain a difficult process. For supports with a high degree of hydrophobicity such as the APS-PHE support a small amount of organic modifier is clearly desirable. Whether it is used in gradient form as described in Fig. 4 or isocratically as described in Fig. 5 appears less important. The use of primary, secondary or tertiary amines as the site of ionic interaction appears more desirable than corresponding quaternary amines. In the former a significant pH effect allows some control of elution volume as shown in Fig. 1 and Fig. 4. Additionally, pH alteration will change the ratio of ionic *vs.* hydrophobic sites where adsorption takes place and thus result in significant selectivity differences.

Nevertheless the present study suggests that anion-exchange supports that have significant hydrophobic character are of value for oligonucleotide resolution.

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