Mixed-Mode Chromatography of Nucleic Acids

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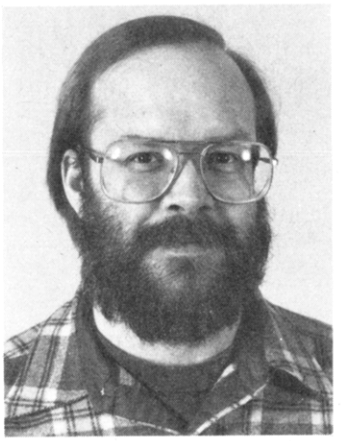

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I. Introduction

Column liquid chromatography is one of the most widely employed techniques for the isolation and purification of a wide variety of biological materials. The process of liquid chromatography involves the interaction of the solute or solute mixture with an insoluble, usually rigid, matrix or stationary phase. The interactions between the solute and the stationary phase can be modulated by variations in the composition of the mobile phase that passes through the column. In an ideal case one solute interacts very strongly with the stationary phase and migrates only slowly through the column while a second solute interacts weakly with the stationary phase and travels rapidly through the column. By maximizing the differences in the interactions that two solutes exhibit with the stationary phase, one can vary their migration velocities through the column such that quantitative separation of both species results.

Different types of chromatographic processes are available to the scientist to solve separations problems. Chromatographic procedures are usually categorized according to the types of interactions available to the solute on the stationary phase. For the resolution of nucleic acids, stationary phases that rely upon ionic interactions (ion-exchange chromatography) or those that employ hydrophobic interactions (reversed-phase chromatography) have generally been the most successful techniques. With the former materials, the stationary phases contain charged residues (cationic sites) for electrostatic interaction with the charged solute (negative phosphodiesters), while the latter materials contain nonpolar residues for hydrophobic interactions with the nonpolar moieties of the solute (nucleobases). Resolution with either of these techniques relies largely upon a single mode of interaction between the solute and stationary phase. Recently, a number of reports have appeared detailing the preparation and use of chromatographic matrices that allow at least two modes of interaction (i.e., ionic and hydrophobic) between the stationary phase and nucleic acid solutes. In such cases resolution occurs via mixed interactions and is known as mixed-mode chromatography. It appears that mixed-mode chromatography employing multifunctional stationary phases can often provide resolution that far exceeds that observed with a single-mode process.

Mixed-mode chromatography is not an entirely new phenomenon. Many chromatographic matrices are based upon a rigid support such as cellulose, agarose, polyacylamide, or silica gel that has been modified to introduce a specific functionality onto the surface of the support. Often the functionality on the surface of the chromatographic matrix and that of the support differ. The solutes introduced onto such a stationary phase experience multiple types of interactions and, in this respect, many chromatographic matrices are in part mixed-mode materials. Free silanol groups on silica gel based matrices are known to affect resolution by reversed-phase chromatography.[1] Hydrophobic interactions alter retention in ion-exchange and affinity chromatographies,[2,3] while electrostatic effects are often present in size-exclusion chromatography.[4] However, these additional, usually secondary, interactions with

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the stationary phase are often viewed as detrimental,[5] and mobile-phase parameters can be designed to optimize the predominant solute–stationary phase interaction while minimizing all others. In only relatively few cases have there been attempts to exploit multifunctional stationary phases and mixed-mode chromatography for the resolution of biological macromolecules. This review will concentrate largely on those reports in which resolution of nucleic acids can be characterized by mixed-mode chromatography. However, prior to a discussion of matrices containing mixed functionality (ionic and hydrophobic), it will be useful to consider briefly ion-exchange and reversed-phase chromatography as applied to nucleic acids.

1. Ion-Exchange Chromatography

Nucleic acids can be resolved by anion-exchange chromatography based upon the interactions of the negatively charged phosphodiesters with cationic sites associated with the chromatographic matrix. The nucleic acid solutes are adsorbed onto these matrices as a result of electrostatic interactions, and desorption occurs by altering the ionic strength of the mobile phase. Retention by such materials in the presence of a mobile phase of increasing ionic strength is usually a function of the number of negative charges (phosphodiester residues) present. With soft gel supports such as agarose, polyacrylamide, and cellulose, alkyl amines have been commonly used to introduce sites for ionic interactions. (Diethylamino)ethyl (DEAE) modified materials are most common and have been successfully used for the isolation and purification of a wide variety of nucleic acids.[6-8] The cationic site of DEAE-based anion-exchange matrices is generated upon protonation of the tertiary amine, and, in this respect, the pH of the mobile phase can have a large effect upon the retention of individual anionic species.

With the advent of porous microparticulate silica gel supports, ion-exchange matrices have been prepared that have resulted in increased resolution for the separation of many biological materials. These silica-based materials are stable at much higher pressures than their soft gel predecessors; thus high flow rates can be employed to reduce the time required for a given separation. The combination of enhanced resolution with reduced analysis time has led to a wide variety of silica-based chromatographic matrices for high-performance liquid chromatography (HPLC).

Silica-based anion-exchange matrices have been produced by coupling primary,[9,10] tertiary,[11,12] or quaternary amines[13,14] to the surface of the silica support. Surface modification is ideally accomplished such that the solute only experiences interactions with the surface-bound bonded phase while the silica gel itself plays no role in the separation mechanism. As with their soft gel predecessors, anion-exchange chromatography of nucleic acid solutes using HPLC matrices relies upon the number of phosphodiester residues available and is largely sequence independent. By selection of the appropriate support pore size, bonded phase, and mobile phase, HPLC anion-exchange chromatography has seen some success for the resolution of short oligodeoxynucleotides,[9,10,13,14] DNA restriction fragments,[11,15-17] plasmid DNA,[15,18] and viral RNA.[19]

2. Reversed-Phase Chromatography

Reversed-phase chromatography was largely undeveloped prior to the introduction of microparticulate silica-based supports. It is "reversed" in that normal-phase chromatography typically involves a polar support (such as naked silica gel) and a relatively nonpolar mobile phase (such as dichloromethane). Polar compounds are more tightly adsorbed by the polar stationary phase than nonpolar ones, and elution of the adsorbed solutes is effected by increasing the polarity of the mobile phase (e.g., addition of methanol to the dichloromethane). In reversed-phase chromatography, as the name implies, the process is reversed. The stationary phase is nonpolar and the mobile phase is polar (usually aqueous). Nonpolar solutes are more tightly associated with the chromatographic matrix than polar ones. Elution of the more hydrophobic solutes occurs as the polarity of the mobile phase is reduced (usually by the addition of an organic solvent such as methanol).

Silica gel based reversed-phase matrices have been prepared by covalently binding a hydrocarbon residue to the surface of the silica support. Ideally, the surface of the silica gel is completely covered such that the chromatographic processes are entirely determined by the character of the bonded phase. Many different residues have been employed for the bonded phase and include, for example, methyl, ethyl, butyl, or octyl moieties. However, the most popular and successful material used to date contains octadecyl (or C_{18}) residues. Separation of nucleic acid mixtures with C_{18} columns typically involves eluting the column with a gradient of methanol, 2-propanol, or acetonitrile in an appropriate aqueous buffer. Reversed-phase chromatography resolves nucleic acid solutes on the basis of their hydrophobic character, largely determined by nucleobase composition, while the length of the solute plays only a secondary role. Reversed-phase chromatography has been successful for the resolution of small oligodeoxynucleotides[20,21] as well as large DNA fragments[22-25] and polynucleotides.[26]

II. Mixed-Mode Chromatography

1. Introduction

With mixed-mode chromatography, resolution relies upon at least two different types of interactions between the solute and the stationary phase. In order to provide solute resolution by mixed interactions, it is necessary that the stationary phases be multifunctional in character.

Many chromatographic materials are multifunctional in that the support is of one character while the bonded phase is of a different character. As noted above, soft gel DEAE-cellulose or DEAE-Sephadex matrices have been often employed for the resolution of nucleic acids. Although they are generally considered to function as anion-exchange materials, the separation of oligonucleotides of the same chain length but differing purine and pyrimidine content has been reported.[27] Further studies have suggested that in addition to ionic interactions, these matrices provide secondary sites for hydrophobic and hydrogen-bonding interactions with the nucleic acid solutes.[28,29] It is this mixed functionality present on the stationary phase that allows for the resolution of the nucleic acid fragments by size (using

electrostatic interactions) and, in part, by nucleobase sequence (using hydrophobic and hydrogen-bonding interactions).

High-resolution silica-based anion-exchange matrices are commonly prepared by the bonding of the cationic residue (amine) to the surface of the silica gel via a short hydrocarbon chain. The carbaceous portion of the bonded phase will commonly give rise to hydrophobic interactions that will alter the retention of solutes resolved by anion-exchange chromatography. In fact, the multifunctional characteristics of ion-exchange matrices are such that it may be impossible to prepare a stationary phase that functions purely on the basis of electrostatic interactions.

Reversed-phase materials are more likely to function exclusively via a single mode of solute–stationary phase interaction in that the bonded phase is composed of a single functionality, a simple hydrocarbon. However, incomplete coverage of the silica gel support with the bonded phase will result in the presence of free silicic acid residues, which have been reported to alter retention mechanisms in reversed-phase chromatography.[1] This can in some cases be reduced if the support is "capped". After coverage of the silica gel with the desired hydrocarbon derivative, the matrix is treated with a small reactive group, such as trimethylsilyl chloride, to mask or cap any residual SiOH functionality.

Although resolution with many types of chromatographic matrices inherently multifunctional in character can be regarded to function as a result of mixed-mode processes, generally the second mode of interaction is a minor effect and can be minimized or eliminated by appropriate choice of mobile-phase parameters. A number of early reports describe the preparation of multifunctional stationary phases, and often these materials resulted in enhanced resolution of nucleic acid mixtures. However, in many cases the mixed-mode nature of the chromatographic process was not clearly recognized. Only recently, with the proliferation of HPLC techniques, have matrices been designed specifically to enhance interactions, of at least two types, between the nucleic acid solute and the stationary phase. Owing to the ionic/hydrophobic nature of nucleic acids, mixed-mode matrices have generally been developed to exploit ionic interactions with the phosphodiesters and hydrophobic interactions with the nucleobase/carbohydrate residues.

2. Early Mixed-Mode Matrices

The direct approach to the preparation of a chromatographic material that contains sites for both ionic and hydrophobic interactions involves modifying a matrix containing one class of residues with a second functionality in order that it take on the necessary multifunctional character. With respect to nucleic acids, one could bind hydrophobic residues onto an ion-exchange matrix or introduce sites for ionic interactions onto a hydrophobic matrix. Both of these approaches have been exploited.

The first approach can be exemplified by work beginning in the 1960s. Mandell and Hershey described a column prepared by converting the carboxylic acid residues of albumin to the corresponding methyl esters and adsorbing this material onto silica gel.[30] Methylated albumin columns of this type were effective in the fractionation of DNA[30,31] as well as soluble RNA and ribosomal RNA.[30-35] Nucleic acids could be resolved on these columns on the basis of molecular size, hydrogen-bonding interactions, and base content. This material was thought to function, with the presence of lysine and arginine side chains, as an anion-exchange matrix, with secondary effects due to the added methyl groups. Gillam et al. extended this idea by adding benzoyl or naphthoyl residues to DEAE-cellulose in order to introduce sites for secondary hydrophobic interactions onto an anion-exchange matrix.[36,37] They suggested that benzoylated DEAE-cellulose fractionated transfer RNAs (tRNAs) partly on the basis of molecular size and partly as a result of secondary interactions between some of the bases of the tRNAs and the benzoyl groups. The elution of some tRNAs from this modified cellulose, particularly those specific for the amino acids phenylalanine and tyrosine, required the addition of some ethanol to the mobile phase. Both tRNAs were subsequently found to contain hypermodified bases of very hydrophobic character in the anticodon loop.[38,39]

RPC-5 columns provide an example of a second method for the preparation of mixed-mode materials. RPC columns are prepared from Plascon 2300, a nonporous spherical polymer (poly(chlorotrifluoroethylene)) that is coated with a solution of Adogen 464 (methyltrioctylammonium chloride).[40,41] In the preparation of this multifunctional phase, the tetraalkylammonium salt was adsorbed onto the hydrophobic beads but not covalently bound as was the case in the previous mixed-mode materials. RPC-5 columns were described as a material for reversed-phase chromatography, based upon the hydrophobic character of the Plascon beads.[40,41] However, the columns were eluted with an increasing gradient of salt, more typical of ion-exchange matrices. The presence of the quaternary ammonium salt adsorbed onto the hydrophobic support produces a material with sites for both hydrophobic and ionic interactions. RPC-5 columns have been effective for the resolution of oligodeoxynucleotides,[42,43] tRNAs,[40,41,44] and DNA fragments.[45,46] The application of RPC-5 columns and the optimization of chromatographic parameters for the resolution of DNA fragments have been described by Wells et al.[47] Although the RPC-5 matrix was remarkably successful for the resolution of nucleic acids prior to the development of the microparticulate bonded phase silica gel materials, it suffered from some drawbacks. The Plascon 2300 support was not of consistent quality and the Adogen 464 coating would bleed from the column at salt concentrations <0.2 M, resulting in a loss of resolution and the necessity of regularly recoating the support. Additionally, the Plascon support is a nonporous material, which reduces the available surface area available for chromatographic interactions (in comparison with a porous support). Large-scale isolations using RPC-5 columns have been reported[48] but generally require columns of large dimensions.

3. HPLC-Based Mixed-Mode Matrices

A. General Considerations

With the advent of microparticulate HPLC-grade silica gel supports, there has been further development and testing of mixed-mode chromatographic matrices.

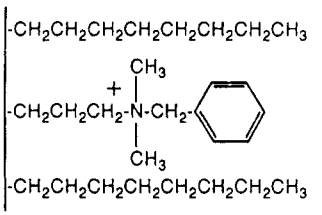
The bonded phase of a silica-based HPLC matrix is commonly attached to the surface of the silica gel by refluxing a suspension of the silica and the appropriate chlorosilane in an inert solvent. After this procedure, the bonded phase is covalently bound to the silica gel support via a siloxane linkage. A number of the reported mixed-mode matrices are composed, in part, of a siloxane-bound phase. One of the inherent problems with these bonded-phase matrices is the characterization of the coverage and functionality present in the bonded phase. This is difficult enough when a single type of functionality is present and becomes increasingly difficult with multifunctional matrices. Elemental analysis gives some indication as to the extent of coverage and, in the case of multifunctional matrices, the ratio of different functionalities present, but this technique is less than ideal. Recently, a procedure has been described for stripping off the bonded phase in hydrofluoric acid and analyzing the resultant fluorosilanes by gas chromatography.[49] This procedure appears to give an accurate determination of the ratio of the functionalities present and, when the surface area of the silica gel is taken into account, allows an estimation of total coverage.[50]

With the preparation of a multifunctional matrix as a potential mixed-mode material, characterization of the chromatographic processes involved, though conceptually simple, is often complicated as a result of the presence and of mixed solute–stationary phase interactions. Although multifunctional matrices often provide enhanced resolution, it is necessary to dissect and analyze the retention mechanisms in order to understand the relationship between mixed-mode and traditional ion-exchange and reversed-phase chromatographies. This process will ultimately lead to the design of more efficient and, if desired, more selective chromatographic materials. Furthermore, the goal of any chromatographic process is the optimization of a separations problem in order that maximum resolution of a complex solute mixture is obtained. Multifunctional chromatographic phases may require complex mobile phase mixtures in order to optimize each type of interaction present. The possibility of examining various stationary-phase compositions, as well as multiple components of the mobile phase, introduces unwanted complexity. A detailed understanding of the mixed-mode process will assist in simplifying the process of optimization involving variations in the stationary- and mobile-phase parameters.

B. Matrix Preparation

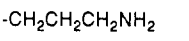
[The illustrations in this section are not meant to accurately portray the nature and/or structure of the various chromatographic matrices but rather give the reader some understanding of the type and variation in the functional groups present in the described multifunctional phases.]

Traditional ion-exchange and reversed-phase matrices have been prepared by using a chlorosilane derivative containing a single terminal functional group. It has also been shown that matrices containing multifunctional phases can be prepared by simply using mixtures of chlorosilanes of differing functionality. Crowther et al. modified a silica gel support using a mixture of chloropropyl- and octyl-derivatized chlorosilanes.[50-52] After this initial step, the chloropropyl groups were subsequently converted to the iodo derivatives and reacted with benzyldimethylamine. This converted the original chloropropyl residues to quaternary amines carrying hydrophobic phenyl groups. The quaternary amines provide sites for ionic interactions while the phenyl groups and octyl groups provide sites for hydrophobic interactions:

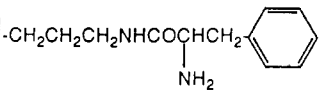


(Aminopropyl)silyl (APS) modified silica gel is a commonly available chromatographic matrix that has been successful for the resolution of short oligonucleotides using electrostatic interactions.[9,10] It functions largely as a pH-dependent ion-exchange material in that the number of sites available for ionic interactions (protonated amines) is dependent upon the pH of the mobile phase. The primary amino group additionally has significant nucleophilic character and is a potential site for covalent modification. Using various activated organic acids that contained a hydrophobic moiety as well as a site for ionic interactions, it has been possible to introduce a mixed ionic–hydrophobic functionality covalently bound to the APS matrix via an amide linkage.[53] A number of materials were prepared that contained phenyl residues as sites for hydrophobic interactions and amines of varying pK_a values as sites for electrostatic interactions:

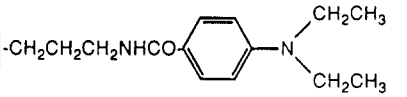
APS



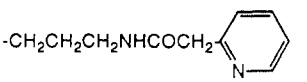
APS-PHE



APS-DEA



APS-PYR



The pH-dependent site for electrostatic interactions was additionally used as a vehicle for optimization of chromatographic resolution via variations in the mobile-phase pH (see "Optimization of Mixed-Mode Chromatography").

In a similar approach, the amino groups of the APS matrix were partially modified with suitable *n*-alkyl carboxylic acids. It was possible to modify about half the primary amino groups as amides carrying a hydrocarbon residue.[54,55] The residual aminopropyl functionality retains the ability to undergo protonation and generate the desired cationic sites. The matrices produced in this manner have pH-dependent sites for ionic interactions (residual amino groups) and sites for hydrophobic interactions that vary with the nature of the *n*-alkyl residue. Initial studies have reported on the character of the matrices containing C_2–C_8 alkyl groups:

APS-C_2

$-CH_2CH_2CH_2NH_2$

$-CH_2CH_2CH_2NHCOCH_2CH_3$

APS-C_4

$-CH_2CH_2CH_2NH_2$

$-CH_2CH_2CH_2NHCOCH_2CH_2CH_2CH_3$

APS-C_6

$-CH_2CH_2CH_2NH_2$

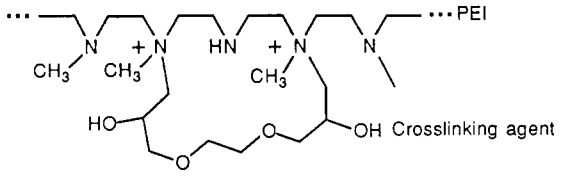
$-CH_2CH_2CH_2NHCOCH_2CH_2CH_2CH_2CH_2CH_3$

APS-C_8

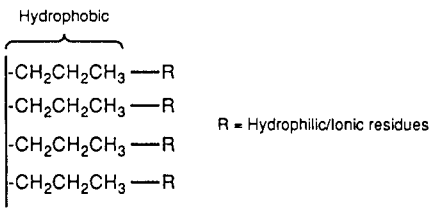
$-CH_2CH_2CH_2NH_2$

$-CH_2CH_2CH_2NHCOCH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_3$

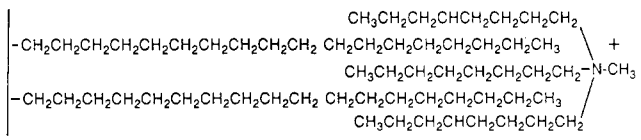
A series of silica-based poly(ethylenimine) (PEI) chromatographic matrices has been prepared by the adsorption of PEI onto the silica support.[56] A chemical cross-linking step stabilizes the multifunctional matrix. Although there are no covalent bonds between the PEI polymer and the silica support, the polymer is essentially irreversibly bound to the support after treatment with the bifunctional cross-linking agent. The amino groups of the PEI provide sites for ionic interactions while the carbaceous portion of the PEI as well as that of the cross-linking agent provide the desired hydrophobic residues. The ionic character of these matrices can additionally be altered by treatment of the cross-linked PEI matrix with alkyl halides to quaternize the PEI amino groups:[57-59]



A two-step procedure described by El Rassi and Horvath[60,61] produced a multifunctional phase that has sites for either weak or strong electrostatic interactions in addition to hydrophobic characteristics. In the first step, propyl groups were covalently bound to the silica surface forming a hydrophobic layer (fur) near the surface of the support. This layer was then further functionalized with either ethylenediaminotetra(2-propanol) or PEI to produce the weak or strong ionic character, respectively:



Two of the approaches described above using an alkylamine matrix involve the covalent introduction of hydrophobic residues to a matrix that is primarily an ion-exchange material in order to produce the desired multifunctional phase. The complementary approach of introducing sites for electrostatic interactions to a hydrophobic or reversed-phase matrix is complicated by the lack of reactive functional groups available on such materials. However, it is possible to coat a suitably hydrophobic HPLC matrix such as the C_{18} materials with a tetraalkylammonium salt such as methyltrioctylammonium chloride in a manner similar to that described for RPC-5 materials.[62,63] The multifunctional matrix prepared by this approach relies upon hydrophobic interactions between the covalently bound octadecyl residues and the hydrocarbon residues of the quaternary ammonium salt to stabilize the matrix:



The quaternary ammonium salt provides sites for electrostatic interactions on a background of hydrophobic hydrocarbon residues.

C. Resolution of Nucleic Acids by Mixed-Mode Chromatography

Resolution by mixed-mode chromatography implies that multiple interactions occur between the solute and the stationary phase, which are in large part responsible for the variations in observed retention times (or volumes) for different solutes. In the case of nucleic acids, mixed electrostatic and hydrophobic interactions have been employed most successfully. When multiple functional groups are available on a stationary phase for interaction with a solute, it is useful to determine if both types of interaction contribute to the retention mechanism. An understanding of the retention mechanisms will simplify the optimization of parameters and allow the design of more efficient matrices. With the APS-modified matrices, it has been possible to observe both electrostatic and hydrophobic interactions by employing two simple types of analyses.[54]

In order for electrostatic interactions to be present on these matrices, it is necessary that the primary amine be protonated, a pH-dependent phenomenon. Decreasing the pH of the mobile phase increases the number of cationic sites present on the matrix and available for interaction with the anionic phosphodiester residues. Conversely, increasing the pH reduces the number of protonated amines available for electrostatic interactions. If electrostatic interactions contribute significantly to the retention mechanism, then one should observe increasing retention, or increasing capacity factor, k' (where $k' = V_r - V_0/V_r$; V_r = solute retention volume and V_0 = column void volume), with decreasing pH of the mobile phase. Analysis of the APS-C_n matrices with a mobile phase of constant salt concentration but varying pH values indicated that larger k' values were obtained (Figure 1) as the pH of the mobile phase decreased.[54] This approach has been successful with nucleic acid solutes because the phosphodiester residues are essentially completely ionized ($pK_a \sim 2$) over the entire pH range commonly employed in the mobile phase. With other biopolymers such as proteins, the ionic character of the protein will also vary with the pH of the mobile phase and further complicate this type of analysis.

Hydrophobic interactions in reversed-phase chromatography are modulated by the presence of varying amounts of organic solvent in the aqueous mobile phase. Increasing the quantity of organic solvent in the mobile

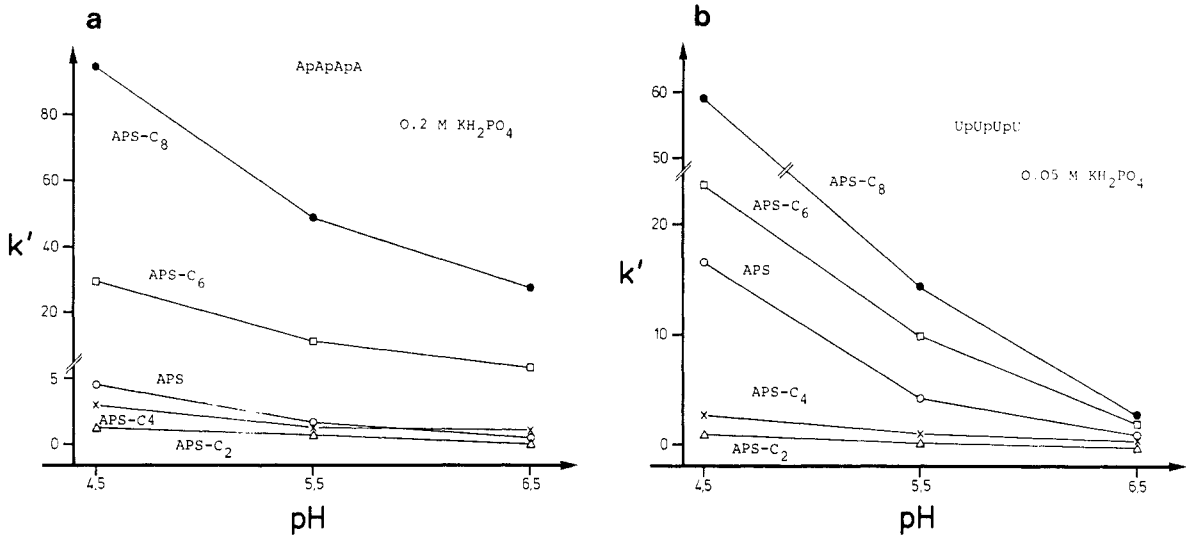


Figure 1. Effect of pH upon the k' values for the solutes (a) ApApApA and (b) UpUpUpU. The k' values were obtained with a mobile phase of 0.2 or 0.05 M potassium phosphate with a pH value of 4.5, 5.5, or 6.5 using the APS, APS-C_2, APS-C_4, APS-C_6, and APS-C_8 matrices. Flow: 1.5 mL/min. Reprinted with permission from ref 54; copyright 1984 Elsevier Science Publishers B.V.

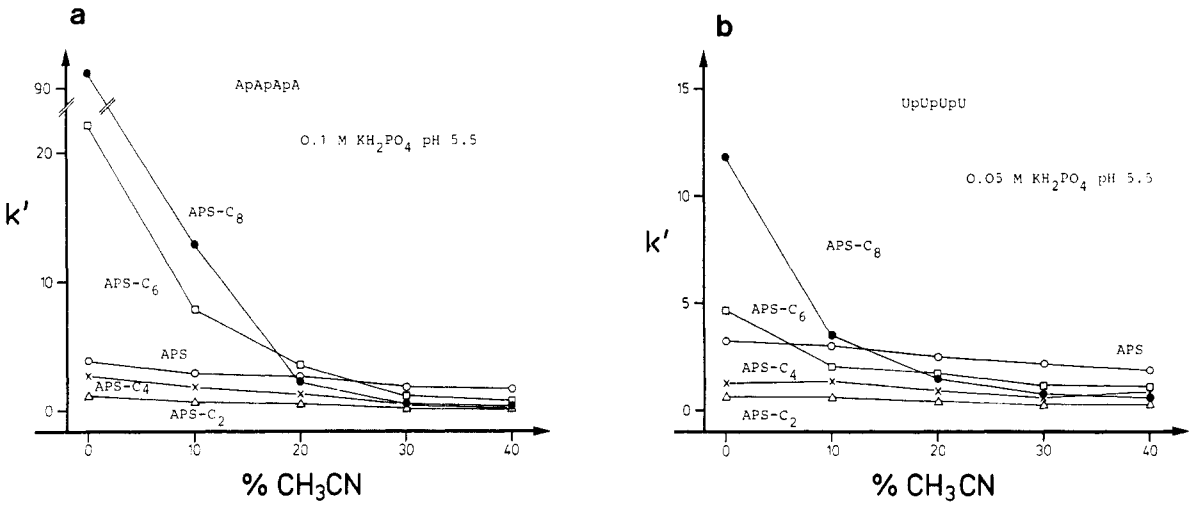


Figure 2. Effect of varying concentrations of acetonitrile upon the k' values for the solutes (a) ApApApA and (b) UpUpUpU. The k' values were obtained with a mobile phase of 0.1 or 0.05 M potassium phosphate, pH 4.5, containing from 0 to 40% acetonitrile using the APS, APS-C_2, APS-C_4, APS-C_6, and APS-C_8 matrices. Flow: 1.5 mL/min. Reprinted with permission from ref 54; copyright 1984 Elsevier Science Publishers B.V.

phase results in reduced retention of the solute by the hydrophobic stationary phase. Similar behavior can be expected for a mixed-mode matrix if hydrophobic interactions contribute significantly to the retention mechanism. The APS-C_n matrices were examined in this respect and they exhibited decreasing k' values with increasing organic solvent concentration (acetonitrile) in the mobile phase (at constant pH and ionic strength) (Figure 2). With the APS-C_n matrices, the k' values also varied with the alkyl substituent as would be expected.[54] In fact, only the APS-C_6 and APS-C_8 matrices exhibited hydrophobic character significantly greater than that of the unmodified APS matrix based upon this analysis. At high concentrations of acetonitrile (>30%), the APS matrix exhibited slightly larger k' values than those observed with the modified supports. This reflects the difference in the density of the sites for ionic interactions. This analysis suggests that with 30% acetonitrile in the mobile phase, the modified materials were functioning almost exclusively via electrostatic interactions. The two test solutes used in this analysis, $(Ap)_3A$ and $(Up)_3U$, differed in hydrophobic character (adenine residues are more hydrophobic than uracil residues), and this difference was reflected in the k' values observed (compare (a) and (b) of Figure 2). This again suggests the importance of hydrophobic interactions in the retention mechanisms for nucleic acids with these matrices.

A mobile phase that allows modulation of both the electrostatic and hydrophobic interactions between the solute and the stationary phase should provide the versatility necessary to exploit any enhancement in resolution available with such multifunctional phases. A gradient of organic solvent concentration, salt concentration, or some combination of the two can be anticipated to resolve nucleic acids differing in hydrophobic character (nucleobase sequence) or charge (length or number of phosphodiester residues). The density of cationic sites present on a multifunctional matrix containing amines will be dependent upon the pH of the mobile phase, which must then be considered as a third mobile-phase parameter. In fact, the generation of a pH gradient during the development of a mixed-mode column could be expected to alter the re-

tention mechanism of charged solutes. At relatively low pH a high density of ionic sites would be present on the stationary phase and the nucleic acid solutes would be tightly associated with the matrix and migrate only slowly through the column. As the pH rises, the number of cationic sites decreases and the solutes will begin to migrate more rapidly. This process should vary with the size (number of phosphodiester residues) of the nucleic acid. Although pH is an important mobile-phase parameter, in practice, the use of pH gradients has not yet proved to be effective in the resolution of nucleic acid materials.[64]

Mixed-mode chromatography to date has typically employed mobile-phase salt gradients, organic solvent gradients, or a mixture of both. Matrices prepared by Crowther et al. using a mixture of chlorosilanes followed by reaction with benzyldimethylamine[51,52] have been eluted with a mixed gradient of salt and organic solvent. For example, a poly(uridylic acid) hydrolysate was resolved to about $(Up)_{16}$ on a column that was composed of 80% octyl residues and 20% quaternary amines using a linear gradient from 0.1 M ammonium sulfate (containing 10% methanol) to 1.0 M ammonium sulfate (containing 35% methanol) in 40 min.[52] From a restriction endonuclease digest of the plasmid pBR322, 22 DNA fragments were obtained, and it was possible to resolve a 267 base pair fragment from one of 234 base pairs using a mixed gradient of KCl (25–250 mM) and methanol (0–30%).[50] The pH of the mobile phases was not reported in this work but may be only a minor consideration when matrices containing quaternary amines are employed. This matrix has also exhibited some success in the resolution of nucleoside and nucleotide materials.[52]

APS-based materials such as the APS-PHE matrix were observed to exhibit maximum resolution with approximately 10% acetonitrile present in the mobile phase [resolution between peaks 2 and 1, R_{21}, is defined as $R_{21} = 2(R_2 - R_1)/(W_2 + W_1)$, where R_2 and R_1 are the corresponding retention volumes and W_2 and W_1 reflect the peak width at base line]. Resolution of nucleic acid fragments with the APS-PHE material employed a mobile phase in which the organic solvent concentration was maintained constant at 10% in the presence of an increasing salt gradient (potassium phosphate) at varying pH values. For example, two complementary undecamers, differing only in one position (containing an adenine or thymine residue), were resolved with the APS-PHE column[53] and a gradient of 0.05–0.9 M potassium phosphate, pH 6.5 (10% acetonitrile).

A gradient of ammonium acetate in the absence of an organic solvent also proved to be an effective mobile phase for the elution of a uridylic acid mixture. Fragments up to about $(Up)_{35}$ in length could be resolved under these conditions (Figure 3).

PEI-based materials have also been effective in the resolution of nucleic acids. The quaternized matrices, which are more ionic in character, have exhibited improved resolution when compared with the nonquaternized matrices.[58] Although these materials have been described as primarily anion-exchange matrices, effective resolution of nucleic acid solutes required the presence of an organic solvent. The organic solvent can be expected to modulate the hydrophobic interactions resulting from solute interaction with the carbaceous

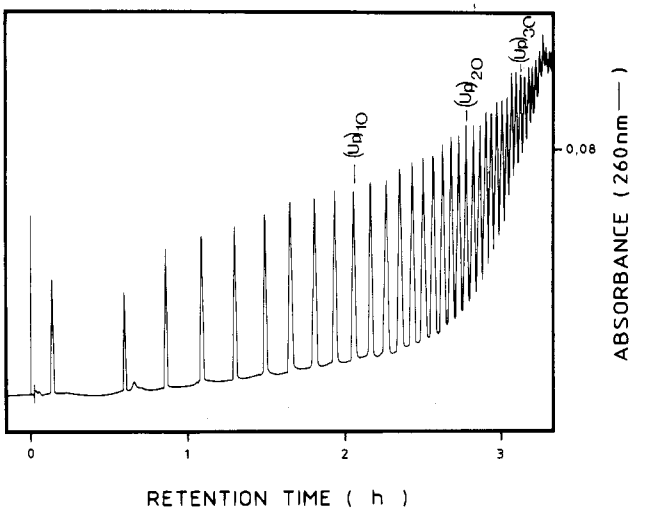


Figure 3. Resolution of a mixture of poly(uridylic acids) on a 4.6 × 250 mm column containing the APS-PHE matrix. Buffer A: 0.5 M ammonium acetate, pH 5.2. Buffer B: 1 M ammonium acetate, pH 5.2. Gradient: 0–100% B in 3.5 h. Flow: 1.5 mL/min.

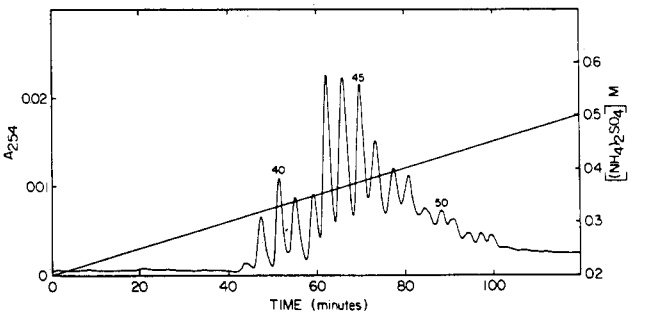


Figure 4. Fractionation of 40–60-base oligodeoxyadenylates on a PEI column. Buffer A: 0.05 M potassium phosphate, 15% acetonitrile, pH 5.9. Buffer B: 1.0 M ammonium sulfate, 0.05 M potassium phosphate, 15% acetonitrile, pH 5.9. Gradient: 0–50% B in 120 min. Flow: 0.5 mL/min. Reprinted with permission from ref 59; copyright 1985 Academic Press.

portions of the PEI matrix. For example, a mixture of adenylic acids varying in length from $(Ap)_{40}$ to $(Ap)_{60}$ could be resolved on a quaternized PEI column[59] using a mobile phase containing 15% acetonitrile and a gradient of 0–0.5 M ammonium sulfate, pH 5.9 (Figure 4).

The materials described by EI Rassi and Horvath[60,61] are examples of one of the few attempts to give the multifunctional stationary phase a specific orientation. In this case the hydrophobic residues were placed very close to the surface of the silica gel support while ionic/hydrophilic residues were placed in a second outer layer. These materials could be eluted with a salt gradient (sodium phosphate, pH 5.5–6.5) in the presence of 2-propanol or decylbetaine (electrostatic interaction chromatography). Under these conditions nucleotides and oligonucleotides could be resolved. These matrices could additionally be used as reversed-phase materials for the elution of a series of alkylbenzenes. In this case an aqueous methanol mobile phase was employed. These analyses indicate the presence of both ionic and hydrophobic interactions during chromatography with these materials.

Some resolution of tRNAs was also observed in the electrostatic interaction mode[61] with matrices containing a poly(ethylenimine) ionic phase. These columns were eluted with a mixed phosphate–decylbetaine gradient (Figure 5).

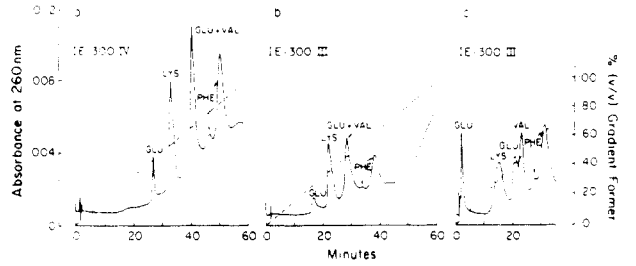


Figure 5. Chromatograms of tRNAs. Columns, 100 × 4.6 mm i.d., 5-μm IE-300 III and IE-300 IV; flow rate, 1 mL/min; temperature, 25 °C. Buffer A: 0.2 M sodium phosphate, pH 6.3 (a and b); 0.25 M sodium phosphate, pH 6.3 (c). Buffer B: 0.5 M sodium phosphate, pH 6.3, containing 5 mM *n*-decylbetaine. Linear gradient in 1 h: (a) 0–100% B; (b) 0–100% B; (c) 0–35% B. Flow: 1.0 mL/min. Reprinted with permission from ref 61; copyright 1985 Elsevier Science Publishers B.V.

The orientation of the hydrophobic phase also plays an important role in the retention mechanisms. Large molecular weight biomolecules appear unable to penetrate the outer ionic/hydrophilic surface while smaller molecules can interact with the hydrophobic surface. With this observation, materials can be designed that do not retain high molecular weight substances but resolve small molecules.[60] This approach would greatly simplify the analytical procedure for physiological samples in that large proteins, which often clog chromatographic columns, would pass harmlessly through while the analysis of drugs or metabolites would continue normally.

Owing to the noncovalent nature of the tetraalkylammonium-coated C_{18} matrices, the use of organic solvents in the mobile phase is precluded, and a relatively high salt concentration must be employed to increase the hydrophobic interactions necessary to stabilize the matrix. These columns have been typically eluted with a gradient of ammonium acetate (0.5–1.5 M). Oligonucleotides obtained from a homopolymer digest could be eluted up to lengths of about 100 uridyl residues (Figure 6) under these conditions.[62,63] This mixed-mode material has also been very effective for the isolation of tRNAs.[63–65] Individual tRNAs are eluted from the matrix in sharp well-defined peaks (Figure 7). A crude mixture of tRNAs containing as many as 90 species could be fractionated into a number of peaks (Figure 8). Some tRNAs were obtained in a purified form after a single chromatographic separation with this matrix. The retention characteristics on this column for tRNAs corresponding to all 20 amino acids, including many isoaccepting species, have been reported.[63]

It is often necessary to use a number of chromatographic steps in the isolation of a specific tRNA. The C_{18}-coated mixed-mode matrix has been very effective as one of the chromatographic steps in such a purification scheme. It also functions well in the analysis of partially purified tRNAs. For example, $tRNA^{Asp}$ (specific for aspartic acid) could be partially purified by chromatography on a soft gel column of benzoylated DEAE-cellulose and further purified by subsequent chromatography on a column of DEAE-Sephadex. The degree of purity of the nucleic acid material collected in either of the purification steps could be monitored by chromatography on the C_{18}-coated HPLC matrix (Figure 9). The material isolated from the DEAE-Sephadex column was purified to homogeneity by chromatography on a preparative (21.2 × 250 mm) column containing the mixed-mode matrix. Subsequent polyacrylamide gel electrophoresis and biological analyses confirmed the purity of the isolated $tRNA^{Asp}$.

D. Optimization of Mixed-Mode Chromatography

Optimization of a chromatographic separation is a necessary procedure that will lead to the unambiguous analysis, identification, or isolation of a given species. This is relatively straightforward with chromatographic processes that are based upon a single model of solute–stationary phase interaction. Most often the mobile-phase parameters are adjusted in order to optimize the rate at which the desired solute travels through the column in relation to other materials in the solute mixture. The process of optimization becomes somewhat more difficult in the case of mixed-mode chromatography. Often a group of stationary phases are available, all of which differ somewhat in the number of ratio of sites for hydrophobic and ionic interactions. Additionally, the mobile phase is usually composed of at least two parameters, salt and organic solvent, in addition to pH, which can all be varied independently. The approach to parameter optimization in mixed-mode chromatography will likely depend upon the specific matrix and whether or not its character is more hydrophobic or more ionic.

Consider, however, one approach to the process of parameter optimization which alters the character of the ionic and hydrophobic interactions experienced by the solute. As noted above, matrices that employ primary, secondary, or tertiary amines as the sites for ionic interactions will be dependent upon the mobile-phase pH for the generation of the cationic sites (protonated amines) on the matrix. With a fixed number of nonpolar residues as sites for hydrophobic interactions, the

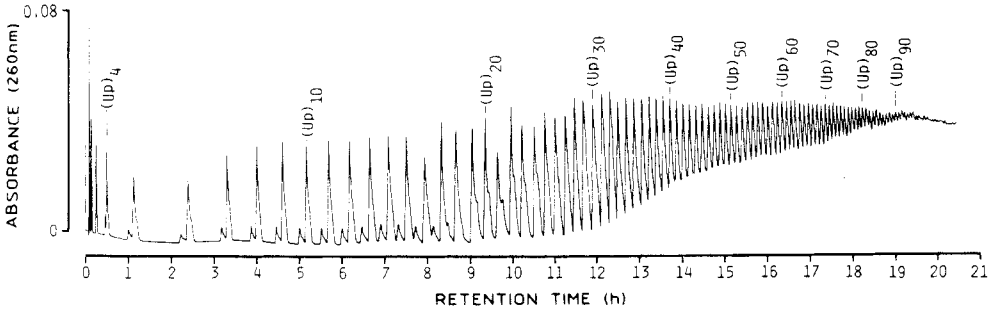


Figure 6. Resolution of 30 A_{260} units of a hydrolysate of poly(uridylic acid) on a 4.6 × 250 mm column of methyltrioctylammonium chloride coated ODS-Hypersil. Buffer A: 0.5 M ammonium acetate, pH 4.5. Buffer B: 5.0 M ammonium acetate, pH 6.0. Gradient: 0–45% B in 20 h. Flow: 0.5 mL/min. Reprinted with permission from ref 63; copyright 1985 Academic Press.

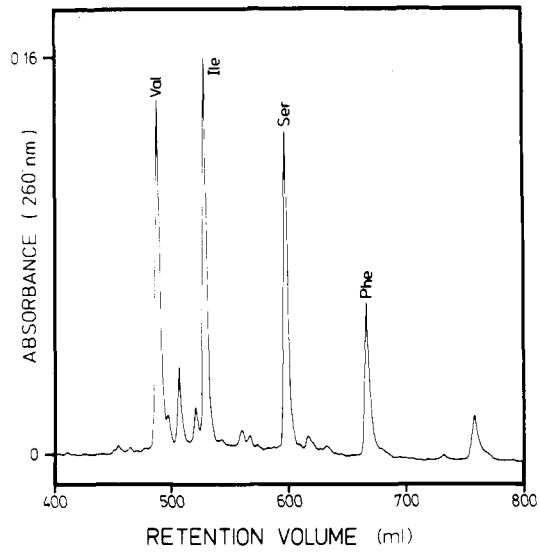


Figure 7. Resolution of four tRNAs specific for the amino acids valine (Val), isoleucine (Ile), serine (Ser), and phenylalanine (Phe). Column and conditions as described in the legend to Figure 6. Reprinted with permission from ref 62; copyright 1983 Elsevier Science Publishers B.V.

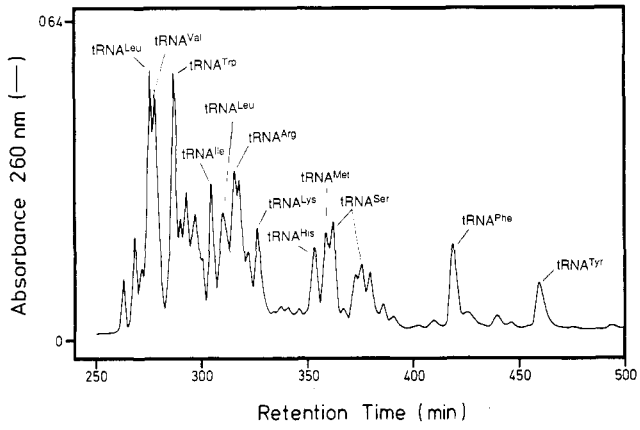


Figure 8. Resolution of a crude mixture of tRNAs. Columns and conditions as described in the legend to Figure 6.

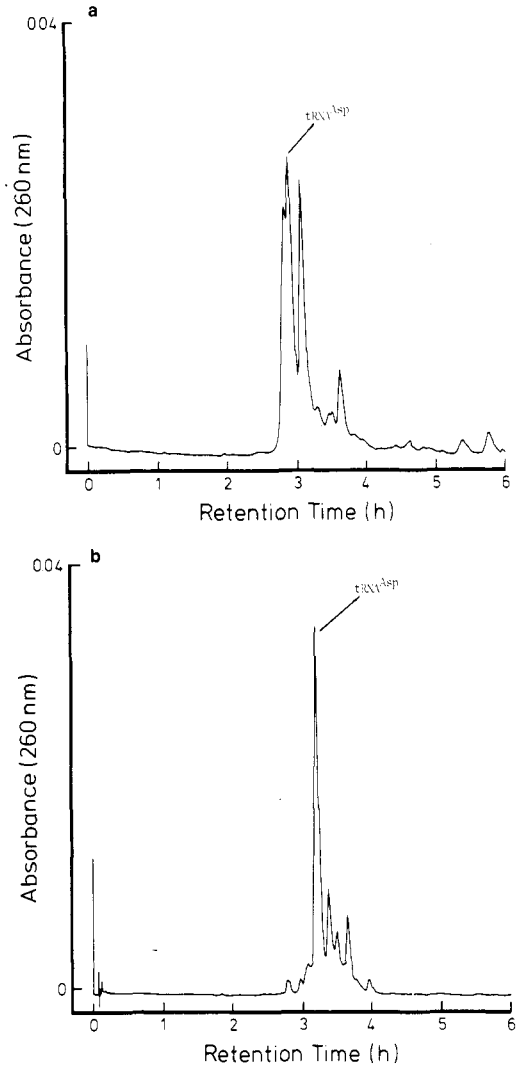


Figure 9. Analysis of partially purified $tRNA^{Asp}$ after chromatography on benzoylated DEAE-cellulose (a) and after chromatography on benzoylated DEAE-cellulose followed by chromatography on Sephadex A-25 (b). Column and buffers as described in the legend to Figure 6. Gradient: 0–40% B in 9 h. Flow: 0.5 mL/min.

number of sites for ionic interactions can be varied with pH. At lower pH values the matrix will then take on a more ionic character (higher ionic/hydrophobic residue ratio), while at higher pH values the matrix will become less ionic (lower ionic/hydrophobic residue ratio). This provides a simple approach to alter the ratio of the hydrophobic to ionic interactions experienced by the solute without the necessity of using a second mixed-mode matrix with a corresponding alteration in the ratio of ionic and hydrophobic residues. For example, a mixture of oligonucleotides containing two hexamers and an octamer was chromatographed[53] on an APS (largely ion exchange) and an APS-PHE (mixed mode) matrix (Figure 10). The APS column could effectively resolve the hexamers from the octamer but resolution between the hexamers was poor (Figure 10a). The APS-PHE column was more efficient in the resolution of all three species, yet some overlap of the peaks for the two hexamers was still present (Figure 10b). The chromatogram of Figure 10b, with a mobile-phase pH value of 5.5, was then repeated at pH values of 6.0 and 6.5 Figure 10c,d). The salt gradient and the concentration of organic solvent remained constant for the last three chromatograms. Two observations can be made from chromatograms b–d of Figure 10. First, the elution time decreased as the pH value increased. This would be expected since raising the mobile-phase pH decreases the number of protonated amines present on the matrix. The salt gradient of the mobile phase remained unchanged so charged solutes should elute more quickly from the column as the density of sites for electrostatic interactions decreases. Second, the resolution between the two hexamers increased as the pH of the mobile phase increased. At higher pH values the matrix should become less ionic such that the hydrophobic interactions begin to play a larger role in the separation mechanism. In this case changing the ratio of the sites for hydrophobic and ionic interactions present on the stationary phase has resulted in an optimized separation of all three species such that base-line resolution is achieved.

III. Processes Related to Mixed-Mode Chromatography

1. Ion-Pair Chromatography

Reversed-phase chromatography of nucleic acids

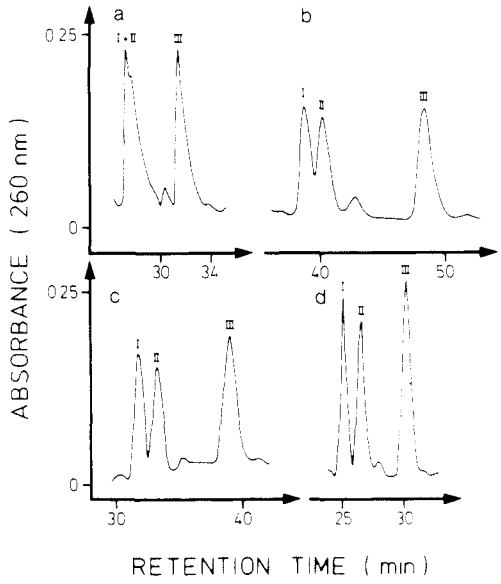


Figure 10. Resolution of the three oligonucleotides ApApUpUpCpGp (I), ApUpUpUpApm^{2}Gp (II), and m^{1}ApUpCpCpApCpApGp (III) on a 4.6 × 250 mm column. Buffer A: 0.05 M KH_2PO_4. Buffer B: 0.9 M KH_2PO_4, 10% methanol. Gradient: 0–100% B in 60 min. Columns: (a) APS matrix, pH 5.5; (b) APS-PHE matrix, pH 5.5; (c) APS-PHE matrix, pH 6.0; (d) APS-PHE matrix, pH 6.5. Flow: 1.0 mL/min.

performed in the ion-paired mode employs a cationic counterion in the mobile phase. The counterion pairs with the anionic phosphodiester residues in order to increase the hydrophobic character of the nucleic acids.[20,66-69] Tetraalkylammonium salts (usually tetramethyl or tetrabutyl salts) are often used as the ion-pair reagent. The ion complex formed in this way exhibits stronger hydrophobic interactions with the reversed-phase matrix (typically a C_{18} material) than does the uncomplexed nucleic acid. The organic solvent content of the mobile phase must be adjusted to compensate for the increased solute hydrophobicity and allow effective elution of the nucleic acid solute. The increased hydrophobic character of the complexed nucleic acid is proportional to the number of phosphodiester residues present or, in effect, the length of the nucleic acid. This technique allows the resolution of nucleic acid mixtures largely by size, with some resolution based upon sequence differences. Ion-pair chromatography as described here has been successful in a number of cases for the resolution of short oligodeoxynucleotides[20,66-69] as well as much longer DNA fragments obtained by restriction endonuclease hydrolysis of high molecular weight DNA.[70]

This approach is similar to mixed-mode chromatography in that ionic and hydrophobic interactions are both employed in solute resolution. However, it differs because the solute–stationary phase interaction is solely one of hydrophobic interactions. The ionic interactions occur only between the mobile phase and the solute, and their primary purpose is to alter the hydrophobic interactions occurring with the stationary phase. Although it may appear that some similarity exists between reversed-phase chromatography in the ion-pair mode and that observed with RPC-5 or tetraalkylammonium-coated C_{18} matrices, the two processes are quite different. The cation in ion-pair chromatography must be continually renewed from the mobile phase and is only transiently associated with the stationary phase. With RPC-5 and coated C_{18} matrices, the tetraalkylammonium salt (methyltrioctylammonium chloride) remains tightly bound to the hydrophobic stationary phase in order to provide sites for ionic interactions. It is not (at least not by design) washed from the column and subsequently renewed by the mobile phase.

2. Hydrophobic Interaction Chromatography

Hydrophobic interaction chromatography (HIC) can include, in a general sense, those aspects of chromatography that have been described here as reversed-phase chromatography or ion-pair chromatography, both of which function primarily as the result of hydrophobic interactions between the solute and the stationary phase as modulated by the presence of an organic solvent in the mobile phase. However, the process of HIC, as it is used today with HPLC matrices, is the development of a technique formerly known as salting-out chromatography. This procedure employs a weakly hydrophobic matrix and a reverse salt gradient; that is, at the beginning of the separation, the salt concentration of the mobile phase is high (~2 M) and it decreases during the development of the column. The high salt concentration is thought to induce interfacial precipitation of the nucleic acid solutes on the stationary phase.[71] Association of the solutes with the stationary phase occurs primarily as a function of hydrophobic interactions. As the salt concentration in the mobile phase decreases, selective resolubilization and elution occur. Small quantities of organic solvents present in the mobile phase can in some cases dramatically alter retention by this mechanism.

Although silica-based materials containing C_2–C_4 alkyl groups are most commonly used for HIC,[71] the PEI,[72] APS-C_n,[55] and conventional soft gel[73-76] materials have been employed with some success for resolution in the HIC mode. In these later cases it is likely that resolution is a function of the hydrophobic residues available with these materials. However, the sites for ionic interaction present with these matrices may contribute to the separation mechanism, if only by altering the hydrophobic character of the matrix. Although this form of chromatography has been most commonly employed for the isolation of polypeptides and proteins,[72] a number of reports have involved the resolution of nucleic acids.[54,55,71,73-76]

3. Mixed-Bed Columns

Mixed-bed columns can be prepared by mixing an appropriate ion-exchange matrix with a reversed-phase matrix.[77] The resulting column will have sites for ionic interactions as well as sites for hydrophobic interactions. This may appear initially as the simplest approach to prepare a column for mixed-mode chromatography. However, at the molecular level a solute traveling through such a column would interact with two types of stationary phases. In one case it would involve only hydrophobic interactions followed by contact with another phase involving only ionic interactions. The mixed-mode materials described in this review have been designed such that a nucleic acid solute experiences ionic and hydrophobic interactions simultaneously. With mixed-bed columns this could occur, in theory, at the interface between two different matrix particles, which is only a very small portion of the

chromatographic surface area available.

IV. Conclusions

The recent explosion in research activity at the academic and industrial levels involving biopolymers has provided techniques that allow the production (and often overproduction) of the desired biomolecules. In order for the products of these techniques to be valuable for further studies, it is equally important that advances in separation and purification techniques match those of molecular and genetic engineering. Mixed-mode chromatography may provide a route to such advances.

The development of new stationary phases suggests that in order to solve a particular separations problem, an entire arsenal of columns should be available for testing. This may be simplified in that some stationary phases can function in more than one mode. For example, some of the materials described in this review can function under one set of mobile-phase conditions largely by electrostatic interactions, under a second set of mobile-phase conditions largely by hydrophobic interactions, and with a third set of parameters by mixed-mode chromatography. Such materials that can employ multiple modes of chromatography depending upon the mobile phase may supply the scientist with a variety of chromatographic procedures but require only a minimum number of columns.

Acknowledgments. I thank former postdoctoral associates, students, and research technicians who have worked with me in this area of separations science applied to nucleic acids. I acknowledge Dr. Rainer Bischoff, who received his Ph.D. for work in the development of mixed-mode chromatography.

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