Journal of Chromatography, 296 (1984) 329–337
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 350

NUCLEIC ACID RESOLUTION BY MIXED-MODE CHROMATOGRAPHY

RAINER BISCHOFF and LARRY W. McLAUGHLIN*
Max-Planck-Institut für Experimentelle Medizin, Abteilung Chemie, Hermann-Rein-Strasse 3, D-3400 Göttingen (F.R.G.)

SUMMARY

Two approaches to the preparation of mixed-mode (ionic–hydrophobic) supports for high-performance liquid chromatography are described. In the first, hydrophobic moieties are covalently bound to an anion-exchange support (APS-Hypersil). In the second approach ionic amines are bound through hydrophobic interactions to a reversed-phase support (ODS-Hypersil). In the former case the hydrophobicity of the support is controlled by addition of alkyl groups of various chain lengths and ionic interactions are controlled by the pH of the mobile phase. The analysis of two simple solutes, $(Ap)_3A$ and $(Up)_3U$, shows that both ionic and hydrophobic interactions are present. In the latter case the support is shown to be useful for the resolution of large oligonucleotides such as $(Up)_{89}$ from $(Up)_{90}$. Hydrophobic interactions are important in the observed separations. The binding of a non-polar amino acid to a particular tRNA results in a large shift in retention volume as a result of a stronger adsorption on the support.

INTRODUCTION

The separation and isolation of nucleic acids has generally been accomplished by means of anion-exchange chromatography using a cellulose or Sephadex matrix with a bound diethylaminoethyl (DEAE) moiety[1–3]. The resolution on these supports is primarily a result of ionic interactions, and oligonucleotides are therefore resolved as a result of the number of phosphate groups available for interaction with the support. Enhanced resolution can sometimes be observed when hydrophobic anion-exchange supports such as benzoylated or naphtoylated DEAE cellulose are employed[4]. RPC-5 chromatography[5,6] is often used to isolate DNA fragments and biologically highly active tRNAs. In this case the solutes are eluted with a salt gradient, but hydrophobic interactions also play a rôle in the observed separations. The development of microparticulate, bonded-phase chromatographic supports and high-performance liquid chromatographic (HPLC) technology allows increased resolution and shorter analysis times for many separations. Anion-exchange as well as reversed-phase supports have been used to separate oligonucleotides[7,8] based on their anionic or hydrophobic properties respectively.

0021-9673/84/$03.00

In the present paper the use of mixed-mode bonded phase HPLC supports is described. They are prepared by two methods. In the first an ion-exchange support is modified to allow additional hydrophobic interactions. In the second a hydrophobic or reversed-phase support is coated with a tetraalkylammonium salt to introduce the desired ionic interactions.

The capacity factors (k') of two different oligonucleotides on the present mixed-mode supports are compared under various pH and organic modifier content conditions. The usefulness of these supports for separating longer oligonucleotides and tRNAs is described.

EXPERIMENTAL

Materials

APS-Hypersil (5 μm) and ODS-Hypersil (5 μm) were purchased from Shandon Southern Ltd. (Runcorn, U.K.), trioctylmethylammonium chloride (Adogen 464) from Serva (Heidelberg, F.R.G.). Reagent grade acetic acid anhydride, butanoic acid anhydride, hexanoic acid anhydride, octanoic acid chloride, ammonium acetate and potassium dihydrogenphosphate were obtained from Merck (Darmstadt, F.R.G.). Acetonitrile (HPLC grade) was purchased from Baker (Deventer, The Netherlands). Bulk tRNA (brewer's yeast) was obtained from Boehringer (Mannheim, F.R.G.). Leucyl-tRNA synthetase (baker's yeast) was isolated according to standard procedures. Poly U was purchased from Sigma (Munich, F.R.G.).

Methods

APS-Hypersil was modified with organic acids of different alkyl chain lengths according to the following procedure. APS-Hypersil (3.5 g, containing 2.1 mequiv. NH_2 groups) was suspended in 15 ml dichloromethane containing 0.5 ml dry pyridine. After the addition of 21 mmol of the corresponding acid chloride or acid anhydride (2.0 ml acetic acid anhydride, 3.4 ml butanoic acid anhydride, 4.9 ml hexanoic acid anhydride and 3.6 ml octanoic acid chloride) the mixture was shaken for 1 h at room temperature. The support was filtered off and washed with methanol (× 2), dichloromethane (× 2), diethyl ether (× 2) and dried under vacuum at 45–50°C. The modified support (100 mg) was then subjected to elemental analysis of C, H and N.

To prepare a sample containing a mixture of oligouridylic acids, 5 mg of polyuridylic acid were dissolved in 1 ml water. Three aliquots of 50 μl were hydrolyzed with 50 μl of 0.3 *M* potassium hydroxide at 37°C for 10, 20 and 30 min respectively. The reactions were stopped at the appropriate time by addition of 2 μl of 6 *M* acetic acid. The three different hydrolysates were then combined and subjected to HPLC analysis.

After resolving the mixture of tRNAs (brewer's yeast), the collected fractions were lyophilized from water several times to remove the ammonium acetate. The residue was dissolved in 50 μl water, and a 40-μl aliquot was used to test for $tRNA^{Leu}$ according to the following procedure. To 40 μl of the desired fraction were added 50 μl of the test solution [0.6 *M* Tris–HCl pH 7.6, 0.4 *M* KCl, 0.02 *M* $MgSO_4$, 2.3 m*M* ATP and 40 μM [^{14}C]leucine (59 mCi/mmol)] and 5 μl of a solution of leucyl-tRNA synthetase (yeast) and the mixture was incubated for 30 min at 37°C. The entire 95-μl

aliquot was spotted on a Whatman No. 3MM filter disk which was washed with 5% trichloroacetic acid (× 3), ethanol and dried. The radioactivity was counted in a liquid scintillation counter using a xylene-based scintillator.

For aminocylation and stabilization of tRNAs specific for leucine in a bulk tRNA sample, 47.5 A_{260} units of bulk tRNA (brewer's yeast) were aminoacylated with [^{14}C]leucine using the test solution described above. The ester linkage between the amino acid and the tRNA was stabilized prior to chromatography according to ref. 9. After HPLC analysis, 100-μl aliquots from each 2.7-ml fraction were spotted on a Whatman No. 3MM filter disk and dried. The radioactivity was counted in a liquid scintillation counter using a xylene-based scintillator.

ODS-Hypersil coated with trioctylmethylammonium chloride was prepared according to ref. 10.

RESULTS

Hydrophobic anion-exchange supports

In order to prepare a series of hydrophobic anion-exchange supports for use in mixed-mode chromatography, an aminopropylsilyl bonded phase silica (APS-Hypersil) was used as a starting material. This support possessed three properties we wished to exploit. As a primary amine, the support functions as an anion-exchange support only in the protonated form. The extent of ionic interactions available to a particular solute will therefore be pH-dependent. Secondly, the three-carbon chain between the surface of the support and the protonated amine should allow some weak hydrophobic interactions. Finally, the primary amine is a nucleophile and can therefore be modified relatively easily.

We have previously reported the preparation and use of some modified supports using primary or tertiary amines which allow ionic interactions and, containing a bound phenyl group, hydrophobic interactions[11]. The most successful of these supports contained a primary amine as the result of coupling phenylalanine to the APS-Hypersil support. In the present approach we decided to modify a specific portion of the primary amines of the APS-Hypersil support. The remaining unmodified primary amines would then provide sites for ionic interaction. In place of the phenyl group for the hydrophobic interactions, alkyl chains of various lengths have been used such that some control of the hydrophobic character of the support would be available. The four modified supports prepared contain two, four, six or eight carbon hydrophobic moieties and are illustrated in Fig. 1. With the described chemical modification (see Experimental), roughly 65% of the primary amines could be consistently modified as measured by elemental analysis.

pH effects

The primary amines of the modified supports should function as pH-dependent anion-exchange sites. As the pH of the mobile phase is increased, the number of sites available for ionic interaction decreases and the solute should exhibit a smaller k' value. $(Ap)_3A$ and $(Up)_3U$ were used as oligonucleotide solutes having equivalent chain lengths (same number of phosphates) but different hydrophobic characters. To determine whether the modified supports functioned in the ion-exchange mode, the two solutes were chromatographed isocratically at three different pH values (Fig. 2a

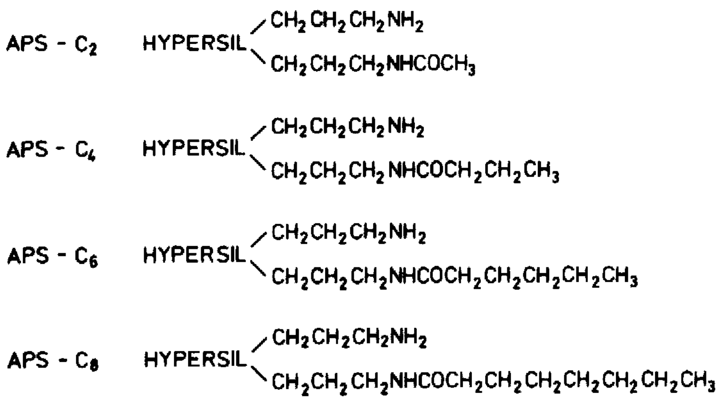

Fig. 1. Structures of the mixed-mode chromatographic supports resulting from modification of 65% of the primary amine groups with acetic acid anhydride (APS-C_2), butanoic acid anhydride (APS-C_4), hexanoic acid anhydride (APS-C_6) and octanoic acid chloride (APS-C_8).

and b). The observation that decreasing k' values result with increasing pH indicates that ion-exchange chromatography occurs with the unmodified APS-Hypersil and all four modified supports. Although $(Ap)_3A$ and $(Up)_3U$ could not be chromatographed under identical conditions, it is still apparent that the k' values for the adenosine-containing solute are in all cases higher than those for the uridine-containing solute. This difference suggests that hydrophobic interactions are also taking place.

Organic modifier effects

In order to confirm that hydrophobic interactions are present, the solutes were chromatographed isocratically in a mobile phase containing various amounts of acetonitrile. Since the organic modifier will disrupt the hydrophobic interactions, a decrease in k' values with increasing concentration of acetonitrile would confirm the existence of such interactions. As seen in Fig. 3a and 3b, the APS-C_6 and APS-C_8

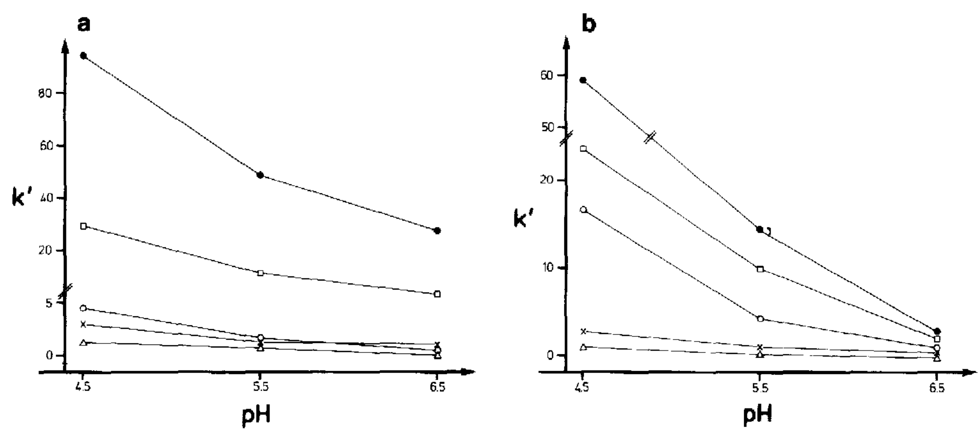

Fig. 2. k' values resulting from the chromatography of (a) $(Ap)_3A$ isocratically using 0.2 M KH_2PO_4 and (b) $(Up)_3U$ isocratically using 0.05 M KH_2PO_4 at pH values of 4.5, 5.5 and 6.5. Supports: △, APS-C_2; ×, APS-C_4; ○, APS; □, APS-C_6 and ●, APS-C_8.

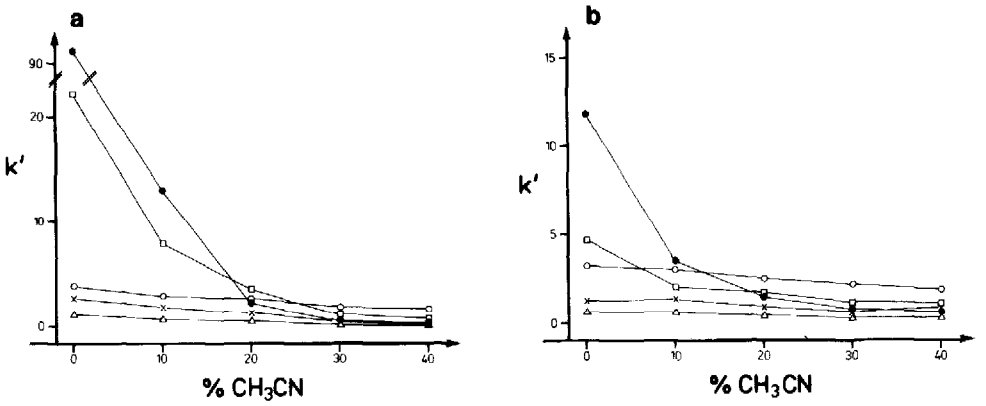

Fig. 3. k' values resulting from the chromatography of (a) $(Ap)_3A$ isocratically using 0.1 M KH_2PO_4 pH 5.5 and (b) $(Up)_3U$ isocratically using 0.05 M KH_2PO_4 pH 5.5 at various concentrations of acetonitrile. Supports are as noted in Fig. 2.

supports result in a dramatic decrease in k' values with increasing concentration of acetonitrile for the solutes $(Ap)_3A$ and $(Up)_3U$. At high concentrations of acetonitrile (30–40%) the unmodified APS support gave the highest k' values.

Ionic reversed-phase supports

In a second approach to prepare a mixed-mode chromatographic support, a hydrophobic octadecylsilyl bonded phase support (ODS-Hypersil) was used as the starting material. Since in this case there was no functional group which could be easily modified, we were unable to prepare a covalently modified support. Instead the ODS support has been coated with either trioctylmethylammonium chloride or trioctylamine[10]. Using the subsequently modified support in the presence of a mobile phase containing a high concentration of salt (ammonium acetate), essentially irreversible hydrophobic binding of the amines to the support occurred. The support appears to function by both ionic and hydrophobic interactions. However, with respect to the resolution of various tRNAs, it is the latter interactions which appear to dominate as we have previously reported[10].

This mixed-mode chromatographic support will also resolve oligonucleotides of relatively high molecular weight. The following example additionally indicates an interesting property of the support. Polyuridylic acid can be hydrolyzed using potassium hydroxide at 37°C. By using three different incubation times (see Experimental) and subsequently mixing the three samples together, one obtains a mixture of oligouridylic acids containing some fragments in excess of one hundred nucleotides in length. While most of the oligouridylic acids contain a 2',3'-cyclic phosphate roughly 5% contain either a 2'- or 3'-terminal phosphate. The mixed-mode column is able to resolve the mixture into a large number of peaks (Fig. 4a and b). In the resolution of solutes eluting relatively early, $(Up)_{10}$ to $(Up)_{25}$, the oligouridylic acids containing the 2',3'-cyblic phosphate can be separated from those solutes containing a 2'- or 3'-terminal phosphate (Fig. 4a). As the oligonucleotides increase in length above $(Up)_{25}$ this resolution is lost. In the latter portion of the chromatogram (Fig. 4b) the resolution of the mixture slowly decreases until fragments longer than $(Up)_{90}$

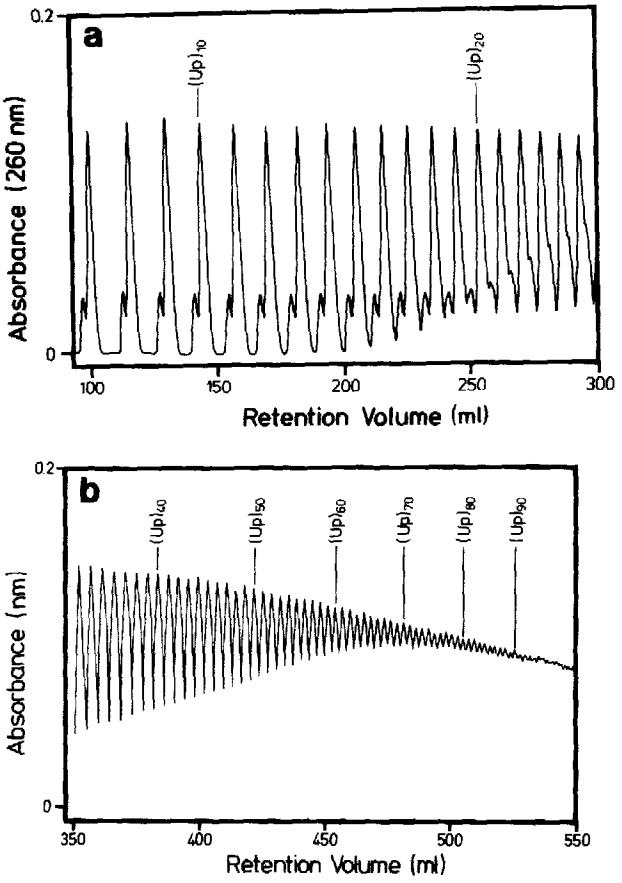

Fig. 4. Resolution of oligouridylic acids from the early (a) and late (b) portions of the chromatogram. The 250 × 4.6 mm column of trioctylmethylammonium chloride coated ODS-Hypersil was used with a gradient including buffer A, 0.5 *M* ammonium acetate pH 4.5, and buffer B, 5.0 *M* ammonium acetate pH 6.0. The gradient was from 100% buffer A to buffer A–buffer B (60:40) in 18 h at a flow-rate of 0.5 ml/min.

are no longer significantly resolved. The support does however discriminate between $(Up)_{89}$ and $(Up)_{90}$. Throughout the chromatogram, very little peak broadening is observed with increasing retention volume, suggesting that little diffusion of the solutes occurs during chromatography.

tRNA isoacceptor separation

The modified support resolves mixtures of tRNAs isolated from brewer's yeast into a number of peaks (Fig. 5a). The collected fractions can be tested for amino acylation activity using the desired amino acid and aminoacyl-tRNA synthetase (see Experimental). Fig. 5a illustrates the analysis of tRNA specific for the amino acid leucine. Since this analysis requires that many fractions are tested for enzyme activity, we examined a second possibility for isoacceptor analysis. Bulk tRNA was aminoacylated with a specific radioactive amino acid and then the chromatographic analysis was repeated and the collected fractions were examined with a scintillation counter

for radioactivity. Unfortunately, the ammonium acetate buffer produces partial hydrolysis of the aminoacylated tRNA. Therefore, the aminoacylation was repeated, but prior to the chromatographic analysis the aminoacyl-tRNA was stabilized by treating the mixture with acetic acid N-hydroxysuccinimide ester[9]. Subsequent analysis indicated that the aminoacyl-tRNA was stable to the chromatographic conditions and peaks could be observed corresponding to the aminoacyl-tRNA. The analysis for tRNA specific for leucine was then repeated by initial aminoacylation of the bulk tRNA, stabilization of the aminoacyl-tRNA and subsequent chromatography on the mixed-mode support (Fig. 5b). In the case of non-polar amino acids a significant shift to a larger retention volume is observed as illustrated for the isoacceptors of Leu-tRNA^{Leu}.

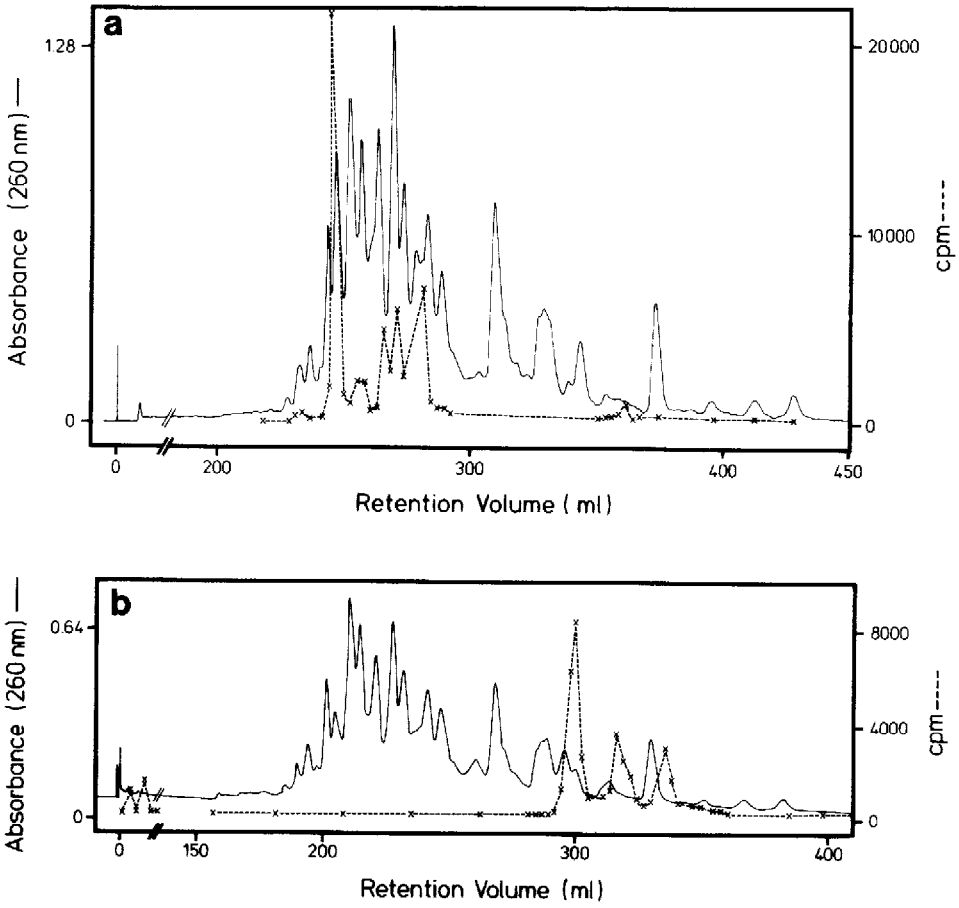

Fig. 5. Resolution of the tRNA isoacceptors for the amino acid leucine from a bulk sample of tRNA from brewer's yeast. Chromatographic conditions as in Fig. 4. (a) Aminocylation activity after resolution of the tRNA mixture; (b) separation of N-acetylleucyl-tRNA^{Leu} from the remaining non-leucine active tRNAs.

DISCUSSION

In the present report we have described the preparation of mixed-mode HPLC supports by essentially two approaches. In the first, a covalently modified support is prepared by modifying a certain portion of the primary amine groups of APS-Hypersil with hydrophobic moieties. Simple alkyl chains of various sizes have been used to introduce the sites for hydrophobic interactions (Fig. 1). The remaining amine groups will allow ionic interactions to occur during chromatography of oligoribonucleotide solutes. The use of a primary amine as the site for ionic interactions means that the extent of ion-exchange chromatography which occurs on the support can be altered by changing the pH of the mobile phase, as is observed (Fig. 2a and b). It is also clear that the hydrophobic interactions occurring on the APS-C_2 and APS-C_4 supports are much weaker than those observed with the APS-C_6 and APS-C_8 supports. The observation that the k' values for the former two supports are less than those observed for the unmodified APS support indicates that the number of sites for ionic interaction has been decreased by the modification and the sites for hydrophobic interaction do not contribute significantly to the observed solute retention. On the other hand, the latter two supports also contain fewer sites for ionic interaction in comparison to the APS support as a result of acylation, but the hydrophobic interactions introduced results in increased k' values. This is confirmed by chromatographic analysis in the presence of an organic modifier. The k' values on the APS-C_2 and APS-C_4 supports are not significantly affected by the concentration of acetonitrile (Fig. 3a and b). There is, however, a sharp decrease in k' values with increasing acetonitrile concentration for the APS-C_6 and APS-C_8 supports. The chromatographic analysis performed in the presence of 40% acetonitrile results in very similar k' values and suggests that retention under these conditions is due solely to ionic interactions. This is also indicated by the observation that at high acetonitrile concentrations the highest k' values for both the $(Ap)_3A$ and $(Up)_3U$ solutes occur on the unmodified APS support where more primary amine groups are present and thus more sites for ionic interaction.

In a second approach to the preparation of mixed-mode chromatographic supports, a reversed-phase C_{18} material has been coated with trioctylamine or trioctylmethylammonium chloride to introduce sites for ionic interaction. In this case a covalent modification was impossible and the modified support is the result of hydrophobic bonding. Under the acidic pH conditions of the mobile phase little difference was observed between the support coated with the tertiary amine and that coated with with the quaternary ammonium salt. The support can in some cases resolve large oligonucleotides resulting from homopolymer digests, as illustrated for oligouridylic acids (Fig. 4a and b). The ability to separate $(Up)_{89}$ from $(Up)_{90}$ appears to be in part a function of the lack of solute diffusion occurring during chromatography. When the peak width for $(Up)_7$ (Fig. 4a) is compared with that of $(Up)_{50}$ or $(Up)_{60}$ (Fig. 4b) very little difference is observed, although $(Up)_7$ is eluted with a retention time of 3 h and $(Up)_{60}$ is eluted with a retention time of 15 h. This implies that the solutes are adsorbed initially very strongly on the support, are released at a specific point in the gradient and that after desorption, they migrate very quickly through the column with a very small apparent k' value.

This mixed-mode HPLC support can also be used to resolve the relatively

complex mixture of tRNAs resulting from a crude extract of brewer's yeast (Fig. 5a). Testing of the eluted fractions indicates that it is possible to resolve a number of isoacceptors for some amino acids as is illustrated for leucine. A second approach available to screen isoacceptor populations is to aminoacylate the bulk tRNA sample with a specific amino acid and stabilize the ester by acylation of the α-amino group. In the subsequent chromatographic analysis the collected fractions are simply counted for the radioactive isotope. Aminoacylation of a tRNA with a non-polar although relatively small amino acid results in a significant shift in retention volume (leucine, phenylalanine, valine). The polar amino acids examined (lysine, arginine and aspartic acid) result in only minor shifts of retention volume. This again indicates the sensitivity of the modified support to hydrophobic interactions. It also suggests, as would be expected, that the 3′ terminus of the tRNA is able to interact freely with the modified support. When the chromatograms of Fig. 5a and b are compared, it can also be observed that the large UV-absorbing peak corresponding to the major leucine isoacceptor as measured enzymatically decreases in intensity (Fig. 5b) as the stabilized aminoacyl-tRNA is shifted to longer retention volume.

CONCLUSIONS

In the separation and isolation of nucleic acids, mixed-mode chromatography offers the possibility of increasing the resolution of a particular mixture. Such resolution may be unobtainable by either ion-exchange or reversed-phase chromatography alone.

ACKNOWLEDGEMENTS

We thank Professor Dr. Friedrich Cramer for his support and interest, Erika Greaser for technical assistance and Dr. Dieter Gauss for careful reading of the manuscript.

REFERENCES

1 Y. Kawade, T. Okamato and Y. Yamamato, *Biochem. Biophys. Res. Commun.*, 10 (1963) 200–203.
2 J. D. Cherayil and R. M. Bock, *Biochemistry*, 4 (1965) 1174–1183.
3 H. G. Zachau, D. Dütting and H. Feldmann, *Hoppe-Seyler's Z. Physiol. Chem.*, 347 (1966) 212–235.
4 I. Gillam, S. Millward, D. Blew, M. von Tigerstrom, E. Wimmer and G. M. Tener, *Biochemistry*, 6 (1967) 3043–3056.
5 A. D. Kelmers, G. D. Novelli and M. P. Stulberg, *J. Biol. Chem.*, 240 (1965) 3979–3983.
6 R. L. Pearson, J. F. Weiss and A. D. Kelmers, *Biochim. Biophys. Acta*, 228 (1971) 770–774.
7 L. W. McLaughlin, F. Cramer and M. Sprinzl, *Anal. Biochem.*, 112 (1981) 60–69.
8 L. W. McLaughlin and E. Graeser, *J. Liquid Chromatogr.*, 5 (1982) 2061–2077.
9 S. Rappoport and Y. Lapidot, *Methods Enzymol.*, 29 (1974) 685–688.
10 R. Bischoff, E. Graeser and L. W. McLaughlin, *J. Chromatogr.*, 257 (1983) 305–315.
11 R. Bischoff and L. W. McLaughlin, *J. Chromatogr.*, 270 (1983) 117–126.