Journal of Chromatography, 418 (1987) 51–72
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3690

REVIEW

RESOLUTION OF RNA USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received January 7th, 1987; revised manuscript received March 13th, 1987)

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1. INTRODUCTION

Ribonucleic acids have generally been categorized according to their functions: messenger RNA (mRNA), ribosomal RNA (rRNA), viral RNA and those involved in the transfer of amino acids to the ribosome (tRNAs). In order to

0378-4347/87/$03.50

understand the interplay and biological roles of nucleic acids, proteins and other molecules, it is often necessary to isolate pure species for detailed study. The amount of material required can vary considerably. The primary sequence of an RNA can usually be obtained with a few micrograms of material, studies involving nucleic acid function can often be performed with a milligram of RNA while detailed structure analysis by high-field NMR or X-ray diffraction requires 10–100 mg of pure material. While the purification of small quantities of RNA (nanogram to microgram range) can sometimes be achieved using polyacrylamide gel electrophoresis, larger quantities usually require an approach involving liquid chromatography. With the recent advances in the technology of stationary phases, high-performance liquid chromatography (HPLC) offers a high-resolution technique for the separation and purification of nucleic acids. Analytical HPLC columns, commonly 250×4.6 mm and containing about 3 g of stationary phase, are employed for analytical separations involving less than milligram amounts of solute. Preparative separations using columns of 250×9.4 mm or 250×21.2 mm and containing 13 or 70 g of stationary phase, respectively, can be used in the milligram to hundreds of milligrams range.

Nucleic acids are in general macromolecular species containing a negatively charged phosphodiester backbone and a variety of non-polar nucleobases. The isolation of pure species can be accomplished by various chromatographic methods. As a result of inherent size, ionic and hydrophobic characteristics one can employ chromatographic methods which rely on a number of interactions: (i) size (gel-permeation chromatography), (ii) interaction with the charged phosphodiesters (ion-exchange chromatography) or (iii) interaction with the carbohydrate/nucleobase moieties [reversed-phase chromatography or hydrophobic-interaction chromatography (HIC)]. All of these approaches have been used with varying degrees of success for both RNA and DNA purification. More recently multiple interactions have been used for nucleic acid resolution. This has been termed mixed-mode chromatography and is a technique based upon at least two types or modes of interaction between a solute and stationary phase (or mobile phase). While this is not an entirely new process it is only relatively recently that chromatographic matrices have been designed to exploit the use of two different chromatographic interactions simultaneously. Owing to the charged (phosphate groups) and hydrophobic (nucleobase/carbohydrate) nature of nucleic acids, matrices have been developed which employ both anionic and hydrophobic interactions. Ideally, it should then be possible to vary the extent of the ionic and hydrophobic interactions experienced by a solute in order that a particular separation problem can be best resolved.

High-performance gel-permeation chromatography has been used in some cases to resolve nucleic acids based upon differences in size [1]. In general, however, chromatography based solely upon differences in size has seen only limited success with nucleic acids.

Anion-exchange chromatography relies largely upon interactions between the negatively charged phosphodiesters and positively charged sites on the chromatographic matrix. This form of chromatography is generally most effective for

small oligonucleotides. However, some modified materials are effective for the isolation of DNA restriction fragments [2] and viral RNA [3].

HIC and reversed-phase chromatography involye non-polar interactions largely between the nucleobases and non-polar groups of the chromatographic matrix. These are easily the most widespread forms of chromatography used at present for the resolution of oligonucleotides and small nucleic acids. They are effective for separations resulting from both size and sequence variations. A second aspect of reversed-phase chromatography is that of paired-ion chromatography. With this procedure an organic counter ion is added to the mobile phase which can pair with an ionic solute. Resolution of oligonucleotides has been successful using triethylammonium, [4] tetramethylammonium [5] and tetrabutylammonium [6,7] ion-pair reagents.

Mixed-mode chromatography has, in fact, been used for some time. Soft-gel ion-exchange resins such as diethylaminoethyl (DEAE)-cellulose or DEAE-Sephadex have been often employed for oligonucleotide and nucleic acid separations [8–10]. They are generally considered to function as anion-exchange resins, however, with DEAE-cellulose the separation of oligonucleotides of the same chain length but different purine–pyrimidine content has been reported [10]. Further studies have suggested that both hydrophobic and hydrogen bonding interactions are involved in this behaviour [11,12]. It is in fact difficult to prepare an ion-exchange matrix which functions solely as a result of ion–ion interactions. There are in general some hydrophobic interactions present unless attempts are made to remove them (for example, the addition of organic solvents such as formamide to the mobile phase [13,14]).

Approaches to the preparation of chromatographic materials for mixed-mode chromatography have generally been two-fold. In the first case an anion-exchange material was covalently modified with hydrophobic moieties to produce a variety of hydrophobic anion-exchange matrices. In the second case, sites for ionic interaction have been introduced into a reversed-phase chromatographic material. Both approaches can produce materials which function as mixed-mode chromatographic matrices.

2. ANION-EXCHANGE CHROMATOGRAPHY

Anion-exchange chromatography of nucleic acids requires that a matrix contain cationic sites for interaction with the anionic phosphodiesters. This can be achieved using a quaternary ammonium salt bonded to the microparticulate silica gel. Such materials are often labeled strong anion exchangers (SAX) such as Whatman-SAX columns. After adsorption of the nucleic acid onto the matrix, selective desorption occurs with an increasing salt gradient (generally sodium chloride or phosphate). Resolution of individual oligonucleotides containing less than twenty base residues is generally possible with SAX materials. In this respect, it is also possible to isolate small quantities of the desired nucleic acid fragment directly from the SAX column.

A second type of anion-exchange matrix contains alkylamines bonded to HPLC-grade silica gel. These materials function as anion-exchange resins when the amine

is in the protonated form. The extent of protonation is dependent upon the pH of the mobile phase. With decreasing pH of the mobile phase, a higher density of cationic sites is generated on the matrix and the oligonucleotides are more strongly bound to the stationary phase. This allows considerable control of retention times for a given solute. At low pH (3.5–4.5) short oligonucleotides (less than ten base residues) are easily resolved. At higher pH values (6.5–7.5) oligonucleotides containing as many as thirty base residues can be eluted from the column.

More recently [15] anion-exchange resins have been prepared by coating HPLC-grade silica gel with polyethyleneimine (PEI) and subsequently cross-linking and quaternizing the amine groups. Using an increasing gradient of ammonium sulfate in an aqueous solution containing 15% acetonitrile oligonucleotides of thirty to fifty base residues can be resolved.

3. REVERSED-PHASE AND HYDROPHOBIC-INTERACTION CHROMATOGRAPHY

Reversed-phase chromatography and HIC are based upon non-polar interactions between the solute and the stationary phase. These chromatographic matrices contain non-polar groups bound to a backbone of silica gel. The most common form of reversed-phase matrix contains C_{18} or octadecylsilyl residues. HIC matrices generally contain shorter carbon chains. With reversed-phase chromatography, oligonucleotides are readily adsorped onto the matrix using a polar mobile phase (aqueous buffer). Desorption occurs as the polarity of the mobile phase decreases, usually by the addition of organic solvent (acetonitrile, methanol or isopropanol). With HIC, nucleic acids are bound at high concentrations of salt (2 *M* ammonium sulfate) to the non-polar stationary phase. Elution of the nucleic acid solute occurs with a decreasing gradient of ammonium sulfate. A second form of reversed-phase chromatography often used for oligonucleotides is that of paired-ion chromatography. An ion-pairing reagent such as a tetramethyl- or tetrabutyl-ammonium salt is added to the mobile phase. The positively charged tetraalkyl-ammonium ion pairs with negatively charged phosphodiester [4–7]. This ion-pairing effect increases the hydrophobic character of the oligonucleotide in proportion to the number of phosphodiesters present or, in effect, the chain length. Resolution in the paired-ion mode can be observed to be a function of both sequence and number of phosphodiesters – in effect the length of the fragment.

4. MIXED-MODE CHROMATOGRAPHY

As the name mixed-mode implies, this form of chromatography employs two types of interactions between the nucleic acid and the stationary phase. In this case, resolution occurs as a result of both hydrophobic and ionic interactions [16]. Two approaches have been used for the preparation of these materials.

4.1. Hydrophobic ion-exchange columns

One technique for the preparation of mixed-mode chromatographic materials involves the modification of an anion-exchange resin with hydrophobic residues

and thus generating a hydrophobic ion-exchange matrix. This was first attempted using soft-gel anion-exchange resins such as DEAE-Sephadex or DEAE-cellulose. After benzoylating or naphthoylating residual hydroxyl groups, enhanced resolution of nucleic acids, primarily tRNAs, was observed [17].

With the advent of HPLC and the corresponding development of porous microparticulate rigid supports, largely silica based, the possibility of developing mixed-mode chromatographic matrices has been reexamined. In a recent report [18] a 5-μm silica was reacted with a mixture of monochlorosilanes. After a two-step procedure a matrix was produced which contained C_8 residues, a quaternary amine and neighboring phenyl residues. Using a mobile phase which allowed a combination of a salt–organic solvent gradient two complementary octanucleotides could be separated and an oligouridylic acids mixture could be resolved to $(Up)_{16}$. In a second report, [19,20], microparticulate silica (3 μm) was coated with PEI and subsequently cross-linked. While this matrix was described primarily as an anion-exchange resin, it is likely that the carbon atoms of the PEI and the cross-linking agent (ethylene glycol diglycidyl ether or butanediol diglycidyl ether) provide sites for hydrophobic interactions. The support has been successful in resolving oligoadenylic acids up to $(Ap)_{35}$ [20]. Our research has taken a slightly different approach to the preparation of hydrophobic ion-exchange matrices. A commercially available aminopropylsilyl (APS) bonded-phase silica (APS-Hypersil) was modified to produce the desired mixed-mode matrix. This material was chosen for a number of reasons. It contained a primary amine group which as a nucleophile should be relatively easy to modify. Anion-exchange resins which contain primary amines will function as pH-dependent anion exchangers. Finally, the propyl spacer between the silica backbone and the primary amine should provide some of the desired hydrophobic interactions. Of the first modified matrices prepared from this material, that which contained a phenylalanyl group bound to the APS material (APS-PHE) proved to be most successful [21].

To determine if mixed-mode chromatography occurs with such materials, that is, whether both ionic and hydrophobic interactions can be observed, the following experiments were performed. Using the APS-PHE material, a solute was chromatographed on the matrix isocratically under various pH conditions. Since the primary amine will act as an anion exchanger only when it is protonated one should observe a decreasing capacity factor (k'), where $k' = (V_R - V_0)/V_0$ (V_R = solute retention volume; V_0 = column void volume), with increasing pH. Using the solute ApApApA, k' values of 22, 11 and 4 were observed at pH values of 4.5, 5.5 and 6.5, respectively, confirming that ionic interactions were present [21]. In the second experiment the ApApApA solute was chromatographed isocratically at pH 5.1 in the presence of varying amounts of acetonitrile. If hydrophobic interactions are available to the solute one should observe decreasing k' values with increasing concentrations of an organic solvent such as acetonitrile. Under these conditions k' values of 83, 28, 15, 10 and 5 were observed with acetonitrile concentrations of 0, 10, 20, 30 and 40%, respectively [21]. With the confirmation that both ionic and hydrophobic interactions were available to the solute, resolution of an oligouridylic acid digest was examined. Using an ammonium acetate

gradient (pH 5.2) the APS-PHE matrix could resolve the uridylic acid hydrolysate up to approximately $(Up)_{35}$.

From a series of hydrophobic anion-exchange resins prepared [21] it was clear that a primary amine functioned well as the site for ionic interaction. A second series of matrices were then prepared in which the APS material was partially modified with *n*-alkyl moieties of different chain lengths [22]. The four modified materials, APS-C_2, APS-C_4, APS-C_6 and APS-C_8, were examined for both ionic and hydrophobic interactions by the methods used for the APS-PHE column. In this case all the materials exhibited ionic interactions when examined isocratically at different pH values (APS-C_6 and APS-C_8 exhibited the strongest pH dependence). Only the APS-C_6 and APS-C_8 matrices also exhibited significant hydrophobic interactions [22]. Application of these last four matrices (particularly APS-C_6 and APS-C_8) for problems in the resolution of nucleic acids is discussed later in this review.

4.2. Ionic–hydrophobic columns

A second approach toward the preparation of useful mixed-mode matrices has involved the modification of a hydrophobic material with a tetraalkylammonium salt to introduce sites for ionic interactions. One of the first chromatographic materials developed in this manner was the RPC-5 support [23,24]. RPC-5 is a reversed-phase chromatographic matrix composed of polychlorotrifluoroethylene beads (Plascon 2300) coated with trioctylmethylammonium chloride. Although chromatography using the RPC-5 matrix is considered by many to be reversed-phase chromatography, the tetraalkylammonium salt coating clearly provides sites for ionic interactions while the polychlorotrifluoroethylene support makes available sites for hydrophobic interactions. The fact that RPC-5 columns are eluted with increasing salt gradients also argues against the material functioning purely as a reversed-phase chromatographic matrix. RPC-5 columns exhibit excellent selectivity for tRNAs [23–25], DNA fragments [26–28] and oligonucleotides [29,30]. There are, however, certain difficulties with the RPC-5 material. The hydrophobic support (Plascon 2300) is no longer commercially available and the material, which can be obtained from various research laboratories, often varies significantly in its ability to resolve nucleic acids. Additionally, the chromatographic support (Plascon 2300) is a non-porous material. While this does not effect analytical uses of the material, preparative isolations often require very large columns. Finally, in our hands, the tetraalkylammonium salt bleeds from the column and requires that the column support to be recoated and repacked at regular intervals.

We have prepared a mixed-mode chromatographic matrix successful for resolution of nucleic acids by coating a silica-based C_{18} modified reversed-phase material (ODS-Hypersil) with trioctylmethylammonium chloride [31]. For this particular reversed-phase chromatographic material a concentration of 300 μmol tetraalkylammonium salt per gram of ODS-Hypersil was optimal [31]. This optimal concentration will vary depending upon the reversed-phase material used and appears to be related to the carbon loading of a particular C_{18} modified silica.

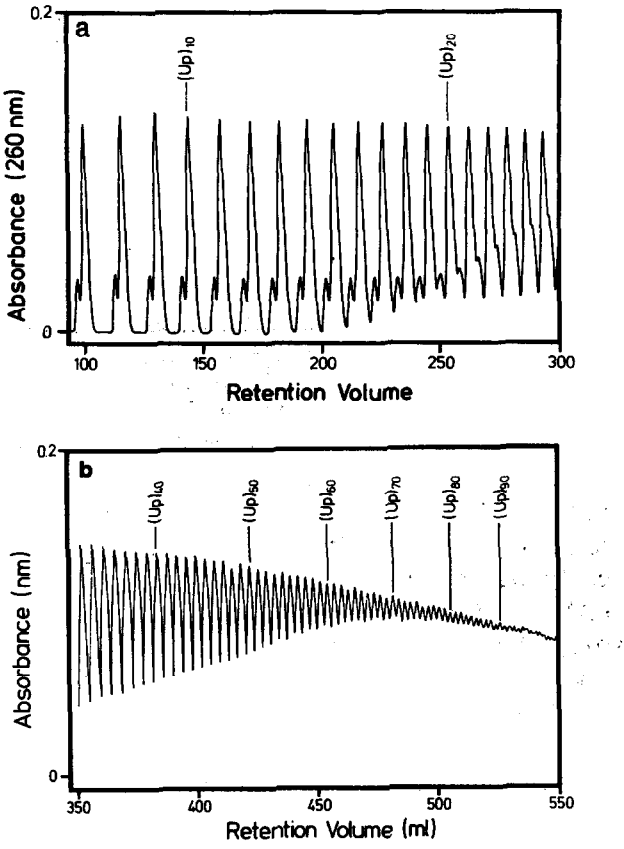

Fig. 1. Fractionation of 30 A_{260} units of a hydrolysate of polyuridylic acid on a 250×4.6 mm column of methyltrioctylammonium chloride-coated ODS-Hypersil. Gradient of 0.5 to 3 *M* ammonium acetate at a flow-rate of 0.6 ml/min. (Reprinted from ref. 31 with permission of the publisher.)

4.3. Resolution of oligonucleotides

We initially examined this mixed-mode matrix for its ability to resolve digests of various nucleotide polymers. The following example additionally illustrates an interesting property of the matrix. Hydrolysis of polyuridylic acid produces fragments most of which contain a 3′-terminal 2′,3′-cyclic phosphate although roughly 5% contain either a 2′- or 3′-terminal phosphate. Using an ammonium acetate gradient and the mixed-mode column prepared by coating ODS-Hypersil with trioctylmethylammonium chloride, the hydrolysate was resolved into a number of peaks (Fig. 1). With solutes eluting relatively early in the gradient, fragments with a cyclic phosphate could be resolved from those containing a terminal phosphomonoester. With fragments larger than $(Up)_{25}$ this resolution was lost. As can be observed from Fig. 1b, resolution of the mixture slowly decreased until fragments longer than $(Up)_{90}$ were no longer significantly resolved. It is noteworthy that throughout the separation illustrated in Fig. 1 very little peak broadening occurred although the fragments longer than $(Up)_{80}$ eluted some 16 h after

the mixture was introduced onto the matrix. It is this effect that accounts for the exceptional resolution power observed. This material has been most effective for the resolution of tRNAs as will be discussed in the following section.

5. RESOLUTION OF TRANSFER RNA

Transfer RNAs are small ribonucleic acids containing between 75 and 90 nucleotides (M_r 25 000–30 000 daltons) with a high percentage of posttranscriptionally modified nucleobases [32,33]. This leads to a variety of 60–90 tRNAs in a living cell that are very similar in size and three-dimensional structure [34–36], which makes their separation particularly difficult.

A number of different methods have been developed for the isolation of specific tRNAs including counter-current liquid–liquid partitioning [37–40], anion-exchange chromatography on carbohydrate matrices containing DEAE moieties [8,9], HIC on weakly hydrophobic agarose or cross-linked agarose stationary phases [41–44] and chromatography on RPC stationary phases consisting of an ion-pairing reagent (methyltrioctylammonium chloride) that is non-covalently adsorbed on a polychlorotrifluoroethylene matrix [23,24]. All of the above mentioned methods except anion-exchange chromatography, separate tRNAs predominantly based upon their hydrophobic properties. This is not surprising, since tRNAs are very similar in ionic character (number of phosphodiesters) but have rather different hydrophobic properties as a result of sequence variations and modified nucleobases.

In the last five years a number of chromatographic methods have been developed for separating tRNAs by HPLC. The techniques are based upon microparticulate porous silica bonded phases, which retain nucleic acids predominantly via electrostatic (anion-exchange chromatography), hydrophobic (HIC) or a combination of electrostatic and hydrophobic interactions (mixed-mode chromatography) as described earlier.

5.1. Separations using anion-exchange chromatography

As a result of similar ionic properties, anion-exchange chromatography is not very effective for tRNA resolution. However, it is possible to enhance the selectivity of anion-exchange supports, especially in the case of tRNAs, by adding sites for hydrophobic interactions. This was demonstrated by two reports describing the use of anion-exchange chromatography for separating tRNAs by HPLC [15,45].

One approach used a stationary phase consisting of an adsorped layer of PEI on microparticulate silica that was subsequently cross-linked into a stable polymeric film with difunctional epoxides as previously described [46]. The amine groups of the PEI layer were finally quaternized with methyliodide to give a strong anion-exchange support [15]. Methylation of the amine groups, however, led to an increase in hydrophobicity of the stationary phase. Thus, it has been shown that the amount of quaternization is an important parameter that must be controlled in order to achieve high resolution of tRNAs and larger oligonucleotides.

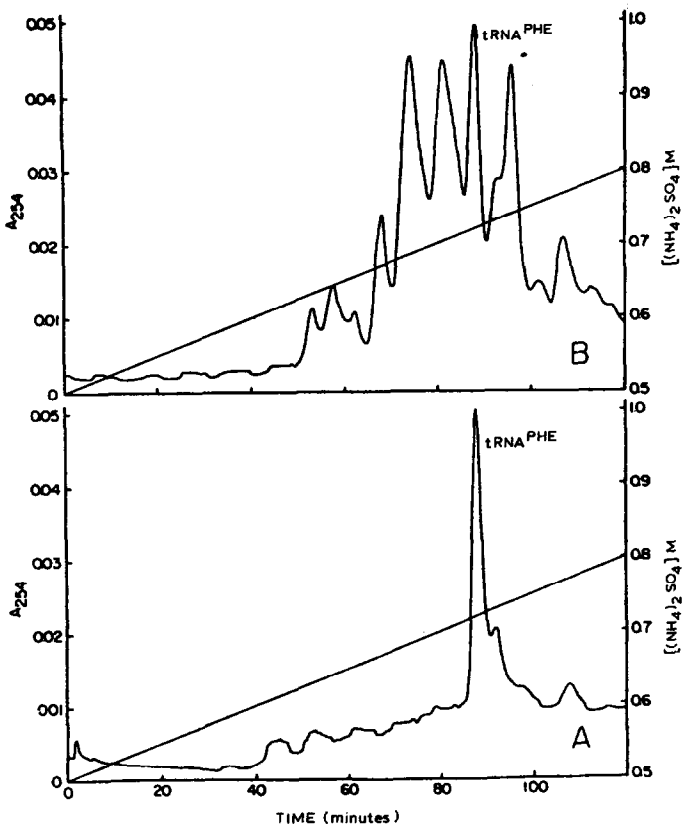

Fig. 2. Fractionation of tRNA from *E. coli* (B) and partially purified $tRNA^{Phe}$ (A) on PEI-coated Hypersil (5 μm, 120 Å pore diameter, 58.8% quaternized); gradient at pH 5.9 with both buffers containing 5% acetonitrile; flow-rate, 0.5 ml/min. (Reprinted from ref. 15 with permission from the publisher.)

As reported, about 60% quaternization appears to be an optimal value. This result indicates the necessity of obtaining a certain ratio between electrostatic and hydrophobic interactions in order to obtain high resolution. In addition the mobile phase can be altered using organic solvents such as acetonitrile which could modulate hydrophobic interactions. As an example, the tRNA separation shown in Fig. 2 was performed with 5% acetonitrile in the eluent.

A second approach to tRNA resolution by anion-exchange HPLC employed microparticulate silica that was derivatized via a two-step procedure resulting in either a weakly or strongly electrostatic anion exchanger [45,47]. In the first derivatization step dimethylpropyl groups were covalently bound to the silica surface forming a weakly hydrophobic layer. This layer was further functionalized with either ethylenediaminotetra(2-propanol) (weakly electrostatic anion exchanger) or PEI (strongly electrostatic anion exchanger) through reactive groups at the end of the propyl chains. tRNAs could be separated on the weakly electrostatic anion-exchanger in either the anion-exchange or hydrophobic-interaction mode depending on the mobile phase conditions (Fig. 3). The pos-

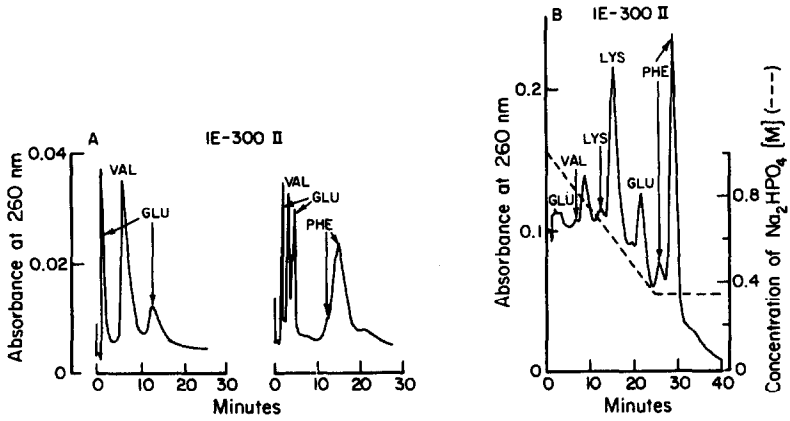

Fig. 3. Chromatogram of specific tRNAs from *E. coli* on ethylenediaminotetra(2-propanol)-derivatized propyl-Vydac (5 μm, 330 Å pore diameter) (IE-300II); eluent, 0.15 *M* disodium hydrogen phosphate pH 6.3. (A) left: containing 10 m*M* Mg^{2+}; right: containing 3.75 m*M* *n*-decylbetaine; (B) gradient of decreasing disodium hydrogen phosphate pH 6.3 as shown in the figure; flow-rate, 1 ml/min. (Reprinted from ref. 45 with permission of the publisher.)

sibility of separating tRNAs by HIC on this weakly electrostatic anion-exchange support indicated that there were hydrophobic interactions present in addition to the electrostatic ones when the support is used in the anion-exchange mode. This was further emphasized by the fact that the most hydrophobic tRNA in the mixture ($tRNA^{Phe}$ from *Escherichia coli*) eluted last (Fig. 3A, right chromatogram). The described results indicate that tRNAs are successfully resolved on anion-exchange matrices containing sites for hydrophobic interactions in addition to the predominant ionic binding sites.

5.2. Separations using mixed-mode chromatography

An approach that specifically employs this phenomenon to improve resolution of tRNAs is mixed-mode chromatography, where ionic and hydrophobic binding sites on the stationary phase are in close proximity to allow both types of interactions to occur simultaneously [16,31,48]. The matrix most effective for tRNA isolation consisted of a C_{18} reversed-phase matrix (ODS-Hypersil, Shandon, U.K.) that was non-covalently coated with methyltrioctylammonium chloride (Adogen 464, Serva, F.R.G.). The final stationary phase proved to be stable over an extended period of time when used with aqueous buffer solutions. Elution of tRNAs was performed with a gradient of increasing ammonium acetate concentration as is generally used in anion-exchange chromatography. Resolution, however, was primarily based upon the hydrophobic properties of the tRNAs. This was demonstrated with two modified tRNAs from yeast (specific for phenylalanine), which differed in either their hydrophobic or ionic properties. The first modified tRNA was prepared by chemically removing the hydrophobic nucleobase wybutosine (Y-base) from the anticodon loop of $tRNA^{Phe}$ ($tRNA^{Phe}_{-Y}$) [49]. This deletion resulted in a significant shift to a shorter retention time (Fig. 4a).

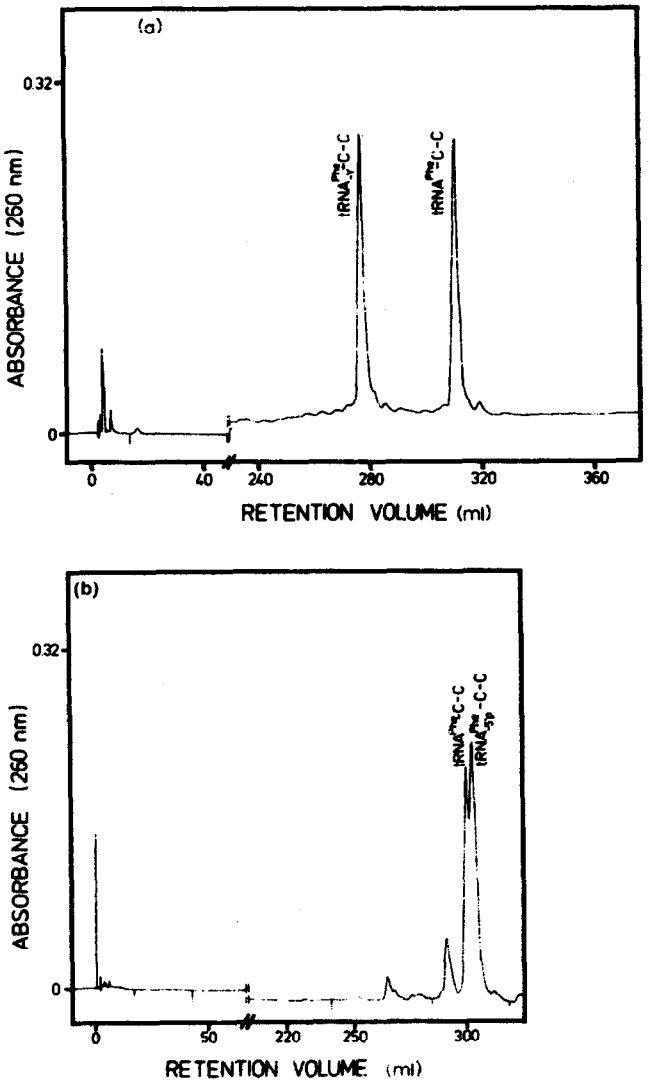

Fig. 4. Mixed-mode chromatographic analysis of two different modifications of $tRNA^{Phe}$ from bakers' yeast; gradient, 0.5 *M* ammonium acetate pH 4.5 to 2.3 *M* ammonium acetate pH 5.3 in 540 min; flow-rate, 0.5 ml/min; resolution of (a) $tRNA^{Phe}$ lacking wybutosine ($tRNA^{Phe}_{-Y}$-C-C) and $tRNA^{Phe}$-C-C; (b) $tRNA^{Phe}$ dephosphorylated at the 5'-terminus ($tRNA^{Phe}_{-5p}$-C-C) and $tRNA^{Phe}$-C-C. (Reprinted from ref. 31 with permission of the publisher.)

The shift is most likely due to reduced hydrophobic interactions between $tRNA^{Phe}_{-Y}$ and the mixed-mode stationary phase, particularly since the modification does not affect the number of negatively charged phosphodiester residues in the tRNA. In a second modification reaction, the 5'-terminal phosphate was enzymatically removed from $tRNA^{Phe}$ [50]. This produced a modified $tRNA^{Phe}$ with one less negative charge than the original molecule. However, $tRNA^{Phe}_{-5p}$ eluted with a slightly longer retention time from the mixed-mode support (Fig.

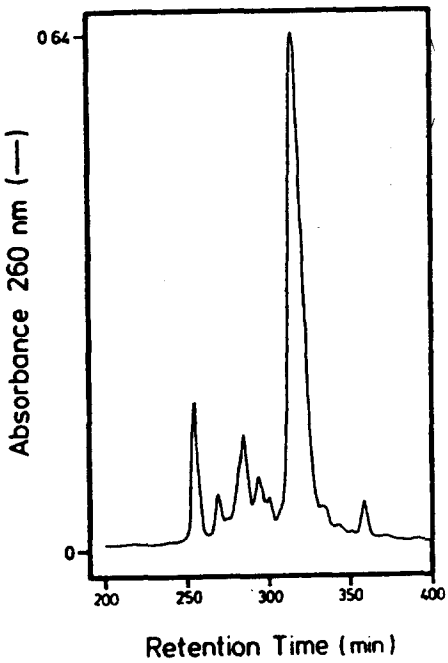

Fig. 5. Resolution of 75 mg (about 1500 A_{260} units) partially purified $tRNA^{Ile}$ from baker's yeast on a 250×21.2 mm column containing the mixed-mode support. Flow-rate, 5 ml/min; other conditions are as described in the legend to Fig. 4. Amino acid acceptor activity before HPLC purification: 0.96 nmol Ile per A_{260} unit; after purification by HPLC; 1.46 nmol Ile per A_{260} unit. (Reprinted from ref. 48 with permission of the publisher.)

4b). This indicates that the reduced electrostatic interactions with the stationary phase are overcompensated by simultaneously increasing hydrophobic interactions as a result of the enhanced hydrophobicity of the 5′-terminus. These experiments show that tRNA resolution on the mixed-mode stationary phase is primarily due to differences in hydrophobic properties.

Mixed-mode chromatography can be effectively employed for isolating milligram amounts of pure tRNAs when used as the final step in an otherwise conventional purification scheme. This was demonstrated for the final isolation of $tRNA^{Ile}$ from baker's yeast after prepurification by several column chromatographic steps on different soft-gel matrices (Fig. 5). Mixed-mode HPLC yielded about 25 mg of $tRNA^{Ile}$ with a significantly higher amino acid acceptor activity.

HPLC on the described mixed-mode support resolved a crude mixture of tRNAs from brewer's yeast into a number of discrete peaks (Fig. 6). After the chromatographic separation fractions were analyzed for their amino acid acceptor activity using ^{14}C-labelled amino acids and the corresponding aminoacyl-tRNA synthetases. A number of isoaccepting tRNA species could be discriminated with this chromatographic matrix. The biochemical role of isoaccepting tRNAs is still a matter of ongoing research, and effective methods for separation and isolation are necessary. Recently a new tRNA activity from chloroplasts has been isolated using mixed-mode chromatography and two-dimensional polyacrylamide gel

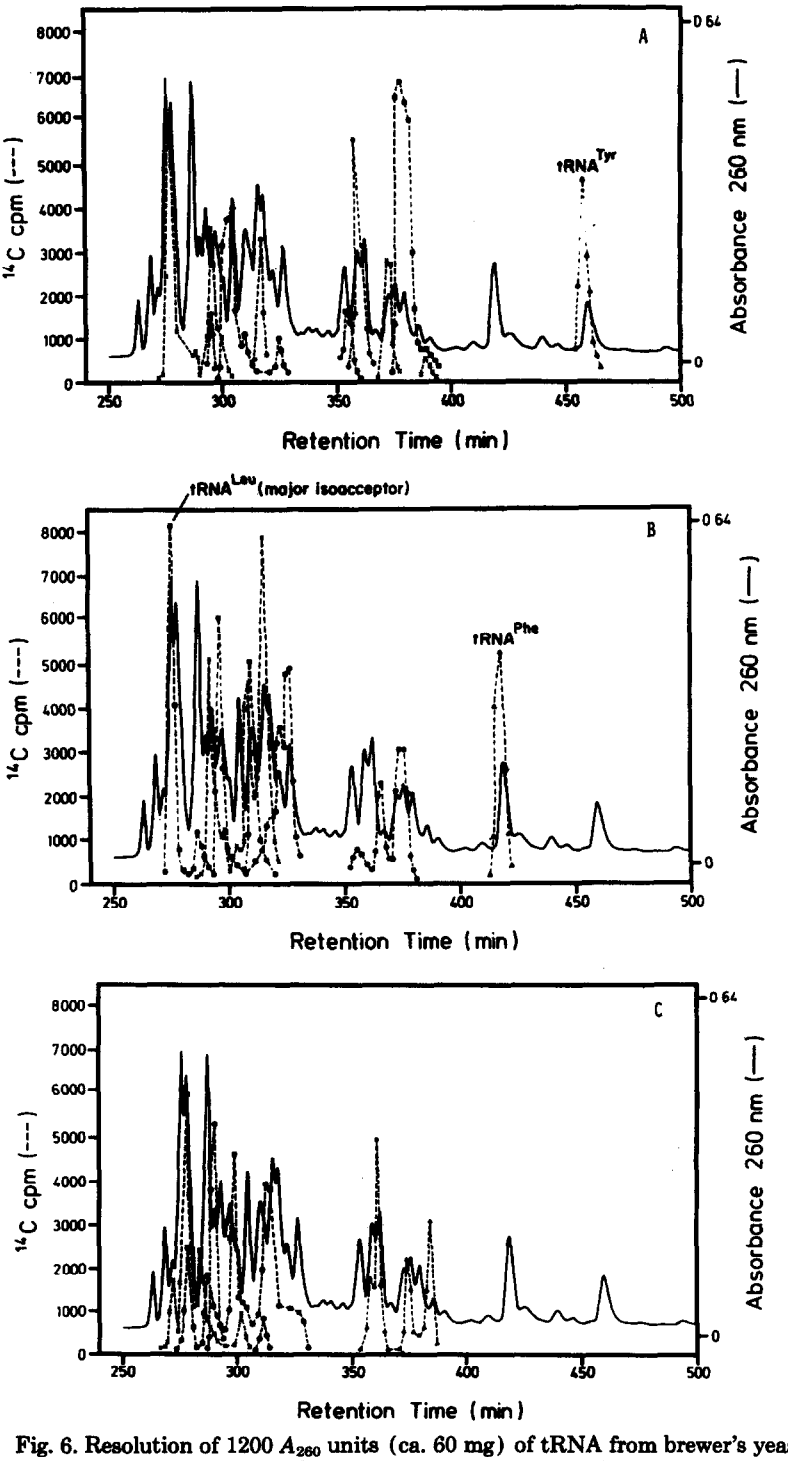

Fig. 6. Resolution of 1200 A_{260} units (ca. 60 mg) of tRNA from brewer's yeast on a 250×21.2 mm column containing the mixed-mode support; flow-rate, 5 ml/min; other chromatographic conditions are as described in the legend to Fig. 4. Aminoacylation activity plotted for the following amino acids: (A) glycine (x), isoleucine (●), methionine (*), histidine (■) and tyrosine (▲); (B) leucine (■), arginine (x), lysine (●) and phenylalanine (▲); (C) valine (x), tryptophan (●), threonine (■) and serine (▲). (Reprinted from ref. 48 with permission of the publisher.)

electrophoresis. This tRNA takes part in the activation of glutamate during the initial step of porphyrin biosynthesis [51,52]. Section 5.4 will describe a purification scheme for an individual isoaccepting tRNA, based upon mixed-mode chromatography and a combination of specific enzymatic aminoacylation and HIC.

5.3. Separations using hydrophobic-interaction chromatography

HIC is a rapid and powerful method for tRNA resolution by HPLC. A number of supports have recently been developed for this purpose [22,53,54]. Separation in HIC is based upon hydrophobic interactions between weakly hydrophobic stationary phases and tRNAs. These interactions are initially induced by a high concentration of ammonium sulfate in the mobile phase and then gradually diminished in a gradient of decreasing sulfate concentration. This results in elution of tRNAs according to their hydrophobic character. An important mobile phase parameter for modulating retention in HIC is the organic modifier concentration. Therefore a small amount of 2-propanol is often included in the mobile phase in order to reduce the affinity of tRNAs for the stationary phase.

One approach to synthesize weakly hydrophobic stationary phases for HIC of tRNAs used microparticulate macroporous silica (Vydac, The Separations Group, CA, U.S.A.) that was subsequently derivatized with trichloroalkylsilanes of varying alkyl chain lengths (C_1 to C_4) [53]. While all of the examined supports resolved tRNAs in the HIC mode, it was the polymeric C_2 and C_4 bonded phases that showed the highest resolution. A crude mixture of tRNAs from *E. coli* was successfully separated into a number of peaks on a polymeric C_2 bonded phase silica matrix with a small amount of 2-propanol (0.75%) in the mobile phase at elevated temperature (55°C) (Fig. 7). Addition of two prepurified *E. coli* tRNAs to the crude mixture indicated that $tRNA^{Val}$ eluted earlier in the gradient than $tRNA^{Phe}$. This is in accordance with their hydrophobic properties, since $tRNA^{Phe}$ is known to be a more hydrophobic tRNA.

In a second set of experiments, weakly hydrophobic stationary phases were prepared by acylating a portion of the primary amine groups of an aminopropylsilyl bonded phase silica (APS-Hypersil), with aliphatic organic acids of varying alkyl chain lengths (C_2 to C_8) [22]. With the described reaction conditions about 65% of the primary amino groups were reproducibly acylated. All supports were suitable for the resolution of tRNAs when used with a gradient of decreasing ammonium sulfate concentration. HIC with APS-Hypersil, which had been derivatized with hexanoic acid anhydride (APS-C_6), resulted in high resolution of tRNAs from baker's yeast as shown in Fig. 8. In this case the mobile phase contained 2% 2-propanol to modulate hydrophobic interactions. The most hydrophobic tRNA ($tRNA^{Phe}$) was eluted last as expected in HIC.

HIC employed with the modified APS supports, like those discussed earlier, can also be used to separate aminoacylated from non-aminoacylated tRNAs when a hydrophobic amino acid is present. Valine-specific tRNA from brewer's yeast was enzymatically aminoacylated with [^{14}C]L-valine using valyl-tRNA synthetase (yeast) as previously described [54,55]. The aminoacylated tRNA was fur-

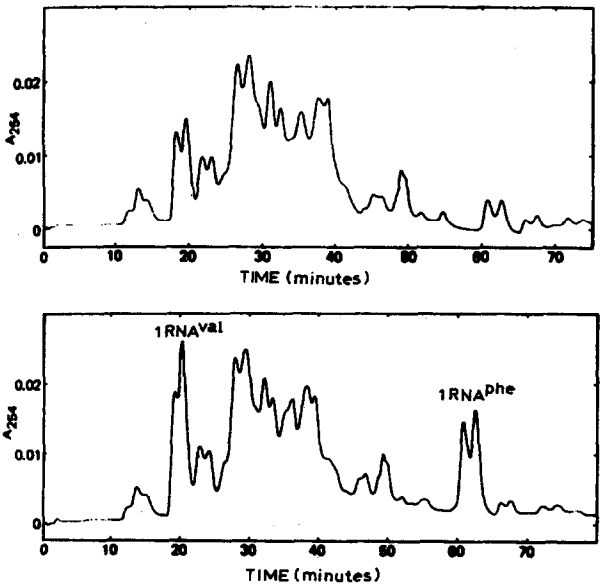

Fig. 7. Resolution of bulk tRNA from *E. coli* on a polymeric C_2 bonded-phase silica support (5×0.41 cm); flow-rate, 0.7 ml/min; gradient: 2.0 *M* ammonium sulfate, 0.2 *M* potassium dihydrogen phosphate pH 7, 0.75% 2-propanol (buffer A) to buffer A without ammonium sulfate in 80 min; temperature, 55°C. Upper chromatogram: 180 μg bulk tRNA from *E. coli* MRE600; lower chromatogram: 180 μg bulk tRNA from *E. coli* MRE600 with added commercially purified $tRNA^{Val}$ and $tRNA^{Phe}$ from *E. coli* MRE600. (Reprinted from ref. 53 with permission of the publisher.)

ther reacted with acetic acid N-hydroxysuccinimide ester according to published procedures [56] which resulted in partial acetylation of the α-amino group of the bound amino acid. Both the aminoacylation with a hydrophobic amino acid and the subsequent acetylation of the α-amino group resulted in chromatographic shifts to longer retention times (Fig. 9a). This is due to an increase in hydrophobicity of the 3′-terminus. The fact that it was possible to separate the aminoacylated from non-aminoacylated species in the case of hydrophobic amino acids without hydrolyzing the labile ester bond between the amino acid and the tRNA allows isolation of at least small quantities of tRNAs that are specific for non-polar amino acids. An example where this specificity was employed for the purification of the major leucine isoaccepting tRNA from brewer's yeast will be described in Section 5.4.

It was, however, impossible to resolve aminoacylated from non-aminoacylated tRNAs by HIC in the case of a hydrophilic amino acid such as aspartic acid (Fig. 9b). In this case acetylation of the α-amino group of the tRNA-bound amino acid was necessary to increase its hydrophobicity such that separation from the non-aminoacylated species occurred. Whether this will provide a general isolation procedure for tRNAs based upon enzymatic aminoacylation and HIC remains to be determined. For example, some of the N-acetylated amino acids are difficult to hydrolyze from the tRNA under alkaline conditions without affecting the tRNA itself [57,58].

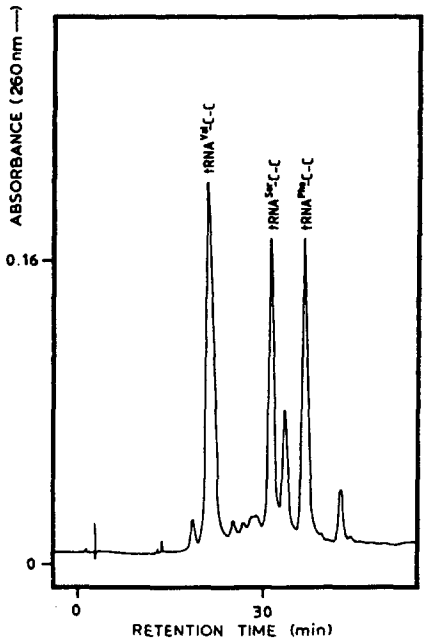

Fig. 8. Resolution of specific tRNAs from baker's yeast on aminopropyl-Hypersil (5 μm, 120 Å pore diameter), where 65% of the primary amino groups are acylated with hexanoic acid anhydride; gradient, 2.0 *M* ammonium sulfate, 0.05 *M* potassium dihydrogen phosphate pH 7 to 0.05 *M* potassium dihydrogen phosphate pH 7 in 60 min (both buffers contain 2% 2-propanol); flow-rate, 1 ml/min. (Reprinted from ref. 22 with permission of the publisher.)

5.4. Purification of a specific tRNA isoacceptor

The following outline of a purification procedure for the major leucine isoaccepting tRNA from brewer's yeast, using both mixed-mode and hydrophobic-interaction chromatography, demonstrates the potential of HPLC in combination with the specific enzymatic aminoacylation reaction for the isolation of small quantities of highly purified tRNAs. In the first purification step crude tRNA from brewer's yeast was fractionated by mixed-mode chromatography. This results in a considerable enrichment of the desired isoacceptor (see Fig. 6B). The fraction containing the major leucine isoaccepting tRNA was subsequently desalted and treated with leucyl-tRNA synthetase (yeast) and [^{14}C]L-leucine to aminoacylate all leucine-specific tRNAs [48,54,55]. At this point of the purification procedure it would be possible to use any of a number of described bioaffinity chromatographic supports which either specifically retain aminoacylated tRNAs in the presence of non-aminoacylated species or vice versa [59–66]. However, in the presence of multiple isoaccepting tRNAs, these methods do not allow the isolation of a unique tRNA, since they are unable to discriminate between different isoacceptors. With the present analysis the reaction mixture, as obtained after the enzymatic aminoacylation reaction, was subjected to a second HPLC purification step by HIC on a C_8 reversed-phase support [octylsilyl (MOS)-

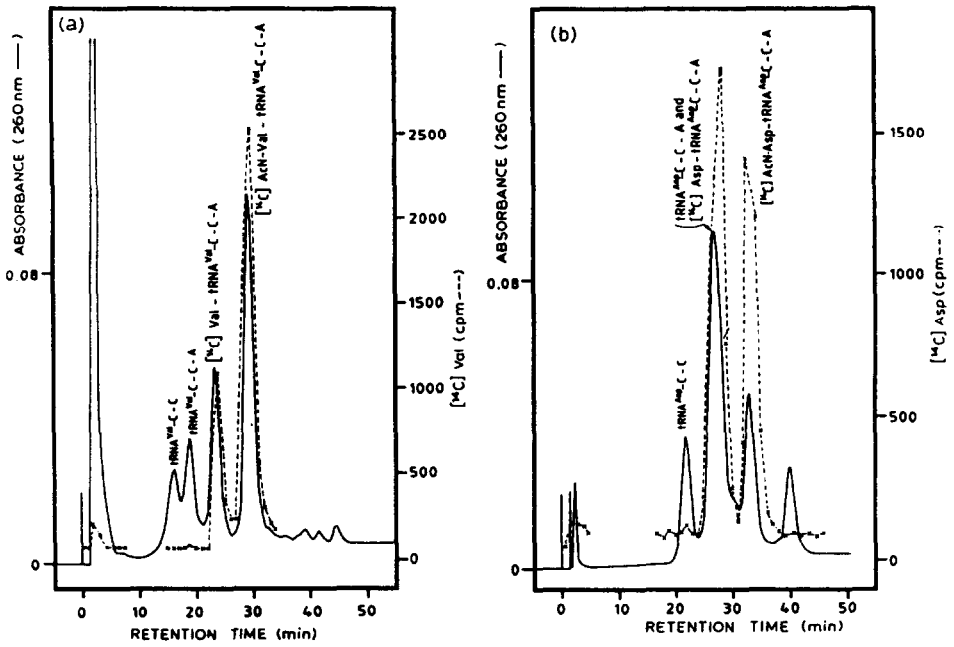

Fig. 9. Resolution of a tRNA mixture resulting from the aminoacylation and subsequent acetylation of (a) yeast $tRNA^{Val}$ and (b) yeast $tRNA^{Asp}$. Chromatographic conditions are as described in the legend to Fig. 8. (Reprinted from ref. 54 with permission of the publisher.)

Hypersil, Shandon, U.K.] (Fig. 10). Measurement of the radioactivity in the collected fractions indicates that two leucine isoacceptors were separated from the remaining tRNAs. In addition to a major leucine isoacceptor a small quantity of a second leucine accepting tRNA was removed during this purification step. Purity of the isolated tRNA was determined by two-dimensional polyacrylamide gel electophoresis [48,67] after removing the bound amino acid by alkaline hydrolysis at pH 9 and subsequent labelling of the 3'-terminus with [^{32}P]pCp and T4 RNA ligase as previously described [68,69] (Fig. 11).

In conclusion HPLC separation of tRNAs is most effective when employing hydrophobic interactions. This can either be achieved on stationary phases containing both ionic and hydrophobic binding sites in close proximity (mixed-mode supports) or with weakly hydrophobic matrices such as used in HIC.

6. HPLC OF OTHER RNAs

In contrast to a considerable number of publications that have appeared on the subject of tRNA separations by HPLC, there have been only a few reports concerning the resolution of other RNAs such as mRNA and viral RNA. This is probably connected with some of the following problems.

(1) Large RNAs like mRNA and viral RNAs are very sensitive to degradation by heavy metal ions and contaminating ribonucleases.

(2) The size distribution of mRNAs is broad, which makes it difficult to isolate

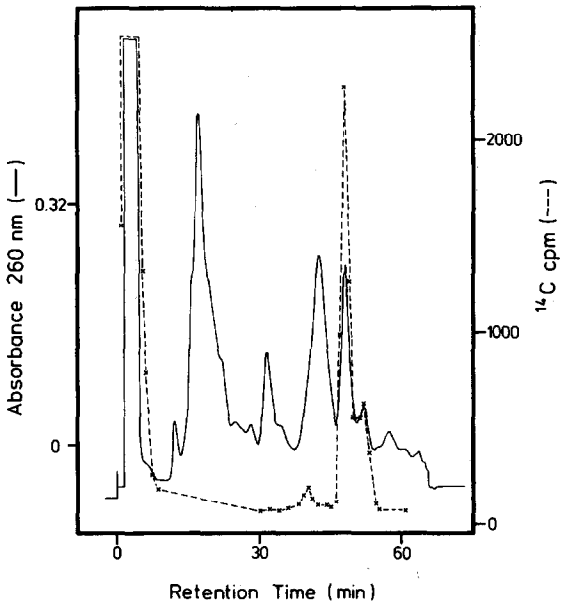

Fig. 10. HIC on a C_8 reversed-phase matrix (MOS-Hypersil) of the reaction mixture obtained by enzymatic aminoacylation of leucine-specific tRNAs in a fraction containing the major leucine isoaccepting tRNA from brewer's yeast after isolation by HPLC on the mixed-mode support (see Fig. 6B). The mixture contains: [^{14}C]Leu-tRNA^{Leu}, [^{14}C]L-leucine, leucyl-tRNA synthetase (yeast), ATP and various unidentified tRNAs. Chromatographic conditions: column, 150×4.6 mm; flow-rate, 1 ml/min; gradient, as described in the legend to Fig. 8. (Reprinted from ref. 48 with permission of the publisher.)

specific mRNAs based upon either size (gel permeation) or ionic properties (anion-exchange chromatography).

(3) A specific message is generally not located on an mRNA of defined size, since variations in either the length of the 5′ leader sequence or the 3′-terminal poly A tail result in considerable differences in size.

Most methods used at present for isolating large RNAs are either based upon certain features of the RNAs like the 3′-terminal poly A tail in mRNAs [70,71] or involve gel electrophoretic separations.

Recently some reports describing the isolation of RNAs by HPLC have appeared. One describes the purification of viroid RNA from tomato plants that are infected with potato spindle tuber virus (PSTV) by using cesium sulfate density gradient centrifugation followed by HPLC on a newly developed anion-exchange support [3]. The anion-exchange matrix was synthesized by reaction of a microparticulate macroporous silica first with (1,2-epoxy-3-propylpropoxy)trimethoxysilane and subsequent opening of the epoxide ring on the silica surface with N,N-dimethylaminoethanol. This resulted in a weak anion-exchange support which binds nucleic acids predominantly through electrostatic interactions. Elution was performed with a gradient of increasing potassium chloride concentration. Single-stranded circular viroid RNA (M_r ~120 000 D) is well resolved from other RNAs such as 4S-RNA (tRNAs), 5S-RNA and 7S-RNA,

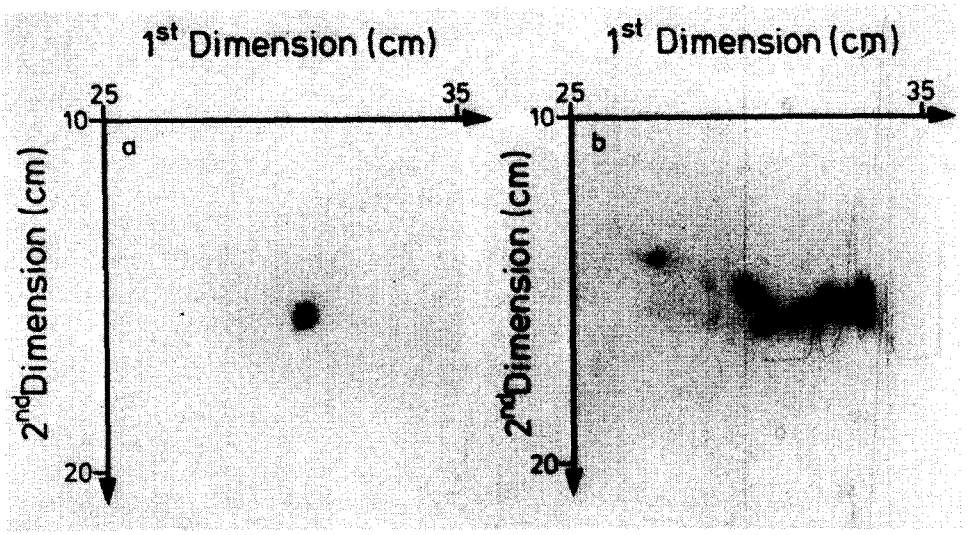

Fig. 11. Two-dimensional polyacrylamide gel electrophoresis of the HPLC-purified major isoaccepting $tRNA^{Leu}$ from brewer's yeast after 3'-terminal labelling with [^{32}P]pCp and T4 RNA ligase. First dimension: 15% acrylamide, 0.35% bisacrylamide, pH 8.3 (Tris–borate buffer) and 7 *M* urea, 50×20×0.1 cm, 1000 V for 24 h; second dimension: 16% acrylamide, 0.35% bisacrylamide, pH 8.3 (Tris–borate buffer) using a stacking gel of 4.75% acrylamide, 0.12% bisacrylamide, pH 6.8 (Tris–HCl buffer), 20×20×0.13 cm, 200 V for 6 h. (a) Major isoaccepting $tRNA^{Leu}$ from brewer's yeast after purification by HPLC; (b) bulk tRNA from brewer's yeast. (Reprinted from ref. 48 with permission of the publisher.)

which cofractionate during the cesium sulfate density gradient centrifugation (Fig. 12). It was noted by the authors that the pore diameter of the silica matrix was an important parameter that has to be optimized to achieve high resolution. In the case of the viroid RNA, 500-Å pores were found to be optimal. Additional precautions were taken to prevent contamination of RNA samples with heavy metal ions or ribonucleases. Therefore all of the wettable stainless-steel parts of the HPLC equipment were electroplated with a 20-μm layer of gold and the stainless-steel tubing was replaced with heavy wall PTFE tubing (pressure stability 20 bar). All buffers were autoclaved. Other authors have reported washing of the HPLC system with an aqueous 0.01% diethyl pyrocarbonate solution prior to fractionation of mammalian tissue mRNAs by gel filtration [72] in order to remove ribonucleases. These examples show that RNA degradation is a serious problem when separating small amounts of large-molecular-weight RNAs by HPLC.

The enrichment of specific mRNAs by gel permeation chromatography on macroporous microparticulate bonded phase silica supports by HPLC demonstrates the potential usefulness of this method [72]. However, to date it does not appear to be a general approach for mRNA isolation.

7. ACKNOWLEDGEMENTS

The authors would like to thank Dr. H. Sternbach (Göttingen, F.R.G.) for providing some of the purified tRNAs as well as tRNA nucleotidyltransferase

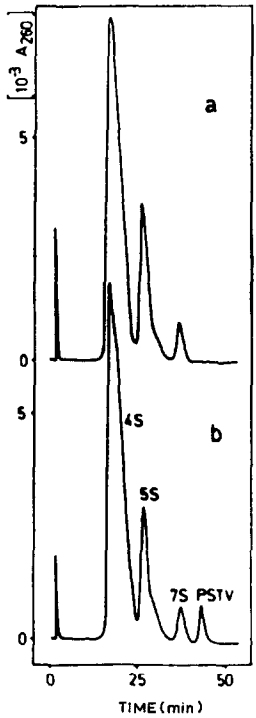

Fig. 12. Analytical HPLC profile of (a) non-infected and (b) PSTV-infected plant RNA extract. RNA (25 μg in 20 μl) was injected into a 40×9.4 mm column containing the anion-exchange support and eluted with a linear gradient from 0.25 to 0.66 *M* potassium chloride in 50 min in 5 *M* urea, 20 m*M* potassium phosphate, pH 6.5, 0.2 m*M* EDTA, at 3 ml/min, 15 bar and ambient temperature. (Reprinted from ref. 3 with permission of the publisher.)

and most of the aminoacyl-tRNA synthetases (yeast). A gift of purified $tRNA^{Asp}$ (brewer's yeast) and aspartyl-tRNA synthetase (yeast) by Drs. R. Giegé and B. Lorber (Strasbourg, France) is gratefully acknowledged. We also thank Mrs. E. Graeser and Mr. K. Hellmann (Göttingen, F.R.G.) for their contributions in preparing modified tRNAs and for performing some of the aminoacylation assays.

8. SUMMARY

High-performance liquid chromatographic techniques can be very effective for the resolution and isolation of nucleic acids. The characteristic ionic (phosphodiesters) and hydrophobic (nucleobases) properties of RNAs can be exploited for their separation. In this respect anion-exchange and reversed-phase chromatography have been successfully employed in the analysis and/or isolation of RNAs. In some cases, particularly tRNAs, chromatographic separations which rely upon multiple interactions between the solute and mobile and/or stationary phases have been highly effective. Mixed-mode chromatography involving simultaneous ionic and hydrophobic interactions, has been employed to resolve complex mixtures of tRNAs. Hydrophobic-interaction chromatography using

gradients of decreasing salt concentration and weakly hydrophobic stationary phases has allowed the resolution of some tRNA mixtures as well as the analysis of modified materials.

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