# Convenient purification of tritylated and detritylated oligonucleotides up to 100-mer

TONYA L. HILL* and JAMES W. MAYHEW

*Beckman Instruments, Altex Division, 4550 Norris Canyon Road, P.O. Box 5101, San Ramon, CA 94583 (U.S.A.)*

ABSTRACT

Oligomers from crude phosphoramidite synthesis mixtures have been purified by reversed-phased high-performance liquid chromatography by exploiting the chromatographic variables of stationary phase pore size, chain length, and gradient shape. Chromatography was performed on oligomers up to 100-mer with mobile phases containing triethylammonium acetate/acetonitrile mixtures. Convenient guidelines are offered to enrich or purify synthetic oligomers.

Tritylated oligomers up to 25 bases in length are best purified on $C_8$ or $C_{18}$, 80 Å columns with moderate strength mobile phases using a combination of isocratic delays and shallow gradients. For oligomers longer than 25-mer, $C_3$, 300 Å columns provide adequate fast purification in as little as 5 min, while 300 Å, $C_8$ columns with long, slow gradients gave substantially increased purity.

Chromatography of detritylated oligomers requires a modified approach. Up to 25-mer they are best purified on 80 Å, $C_{18}$ columns with much lower organic concentrations and shallower gradients than those used for tritylated oligomers. Detritylated oligomers greater than 25-mer can be enriched on both $C_3$ and $C_8$, 300 Å columns using the same conditions described for shorter detritylated oligomers.

INTRODUCTION

Successful oligonucleotide chromatography requires a thoughtful assessment of analytical choices in light of experimental goals. Before the synthesis is even begun, the scientist chooses the oligomer base sequence and length, and whether the products of synthesis are to be recovered in the tritylated or detritylated form. Other variables affect the separation of the products during the chromatographic purification. These include pore size, ligand chain length, gradient shape and selection of solvent type and ion-pairing agent[1–4]. Both the synthesis and chromatographic variables are interactive. An understanding of these interactions is important so that the required level of product purity can be achieved conveniently.

The base sequence and length of the oligomer are of course defined before synthesis. The effect of base sequence on the purification of oligomers has been previ-

ously studied, and no absolute relation was found between oligomer length or base sequence and capacity factor ($k'$) (ref. 5). The choice of whether to recover the oligomers in the tritylated or detritylated form is determined by the degree of purity and speed of analysis required. Generally, the scientist will choose the detritylated form if he is interested in isolating an enriched fraction containing his oligomer, requires a fast clean-up, or desires a quality assurance check on a routine synthesis.

The tritylated from will be chosen for the separation of very-high-purity product, or for preparative work[6,7]. Such applications will require the removal of some or all of the following impurities: synthesis by-products, truncated chains, base modified chains and chain cleavages[8]. If purified in the trityl-on form, the trityl group must be hydrolyzed and extracted after chromatographic purification. An additional purification step may even be required for some specific applications[9].

Pore size, ligand chain length and gradient shape are the primary tools for the reversed-phase high-performance liquid chromatography (HPLC) of synthetic oligonucleotides. Our studies include the chromatography of both tritylated and detritylated oligomers over the range 16- to 100-mer. Studies were performed on stationary phases with 80 Å pore diameters ($C_8$ and $C_{18}$) as well as 300 Å stationary phases ($C_3$ and $C_8$). Convenient guidelines are offered to enrich or purify:

  (1) tritylated oligomers up to 25-mer;
  (2) tritylated oligomers greater than 25-mer;
  (3) detritylated oligomers up to 25-mer; and
  (4) detritylated oligomers greater than 25-mer.

EXPERIMENTAL

*Instrumentation*

HPLC analysis was performed with a Beckman System Gold™ for Methods Development consisting of a Model 126 programmable solvent module, a Model 167 programmable scanning detector module monitoring at 254 nm, a Model 210A injection valve with a 20-$\mu$l loop, and an IBM-AT personal computer with System Gold software.

*Chemicals*

Acetonitrile was HPLC grade from J. T. Baker (Phillipsburg, PA, U.S.A.). Sequanal grade triethylamine used for preparation of the buffers was purchased from Pierce (Rockford, IL, U.S.A.). Buffers were prepared from high-purity water (Milli-Q). Glacial acetic acid was obtained from Mallinkrodt (Paris, KY, U.S.A.).

*Chromatographic conditions*

Gradient elution was used for the reversed-phase separations. Gradient profiles are given on each chromatogram. Various gradients and isocratic delay times were used for the separations as described below.

The aqueous buffer was 0.1 $M$ triethylammonium acetate (TEAA), pH 7.0. The working solutions were prepared by dilution of a 0.5 $M$ stock. The stock was prepared as follows: 27 ml of glacial acetic acid was added to 850 ml of HPLC-grade water. A 70 ml volume of sequanal-grade triethylamine was added with stirring. The pH was adjusted to 7.0 ± 0.05 with glacial acetic acid, and then the solution was brought up to 1.0 l.

The flow-rate was always 1.0 ml/min. Mobile phases for all chromatography are: (A) 0.1 M TEAA, pH 7.0; (B) 0.1 M TEAA in 70% acetonitrile.

A description of the methods follows.

*Method 1:* 3 min at 15% B, increase to 33% B over 11 min, isocratic for 29 min, then to 100% B over 4 min.

*Method 2:* increase from 15% B to 29% B over 10 min, isocratic for 20 min, then to 100% B over 4 min.

*Method 3:* increase from 15% B to 29% B over 3.3 min, isocratic for 6.6 min, then to 100% B in 1.32 min.

*Method 4:* 1 min at 15% B, increase to 57% B over 1.6 min, isocratic for 3.65 min, then to 15% B in 0.66 min.

*Method 5:* 3 min at 15% B, increase to 40% B over 5 min, isocratic for 4 min, then to 57% B over 2 min.

*Method 6:* increase from 0% B to 29% B over 40 min.

*Method 7:* increase from 0% B to 29% B over 12 min.

*Method 8:* increase from 0% B to 21% B over 60 min.

*Samples*

All oligomers were chromatographed in their crude form and were not extracted or desalted in any way before analysis. All oligomers were gifts and were synthesized by the phosphoramidite method. The sequences of the crude oligomers follow.

16-mer 5′ TGC TCT TGT TGA GCA G 3′

17-mer 5′ TGC TCT TGT TGA GCA GT 3′

29-mer 5′ GGC CAG TGC CAA GCG TGC TAG CCT GCA GG 3′

33-mer 5′ GAT CCC AGA AGT AGT TTT GAT GCA CAT GCA ACG

36-mer 5′ GCT TAG AAA GGA GGT GAT CCA GGT TTT GAA TTC
         ACA 3′

37-mer 5′A GCT TAG AAA GGA GGT GAT CCA GGT TTT GAA TTC
         ACA 3′

76-mer 5′CCT TCA GTT CAG GGG ACA GCT CCT TGG TTC TTC CAT
         ACA GGG TAA TTT TGA AGT ATT GCT TGT TTT CAG TTC
         AGA A 3′

100-mer 5 ′ AGC AGC AAG CTT GGT CGA CAG ATC CAG GAG AGG
          TCA ACC TGT CTG GTC TGA CTG TCT GCT CCC TTC TGA
          TCA ACC TAG GTT TGG GGC AGA GTT TGA TCT G 3′

Sample load was 5 to 50 μg of each oligomer in an injection volume of 5–20 μl. All samples were diluted to the same composition as the starting mobile phase of the gradient to be used.

When necessary, certain oligomers were detritylated. These samples were reduced to dryness under vacuum, then resuspended in 1.0 ml of 80% acetic acid. The solution was held at room temperature for 45 min and was then dried under vacuum. The residues were resuspended in the starting mobile phase. No further extraction or desalting steps were performed.

RESULTS AN DISCUSSION

*Tritylated oligomers*

The purification of tritylated oligomers will be discussed before the purification of detritylated oligomers. In each case we will illustrate the contributions of three chromatographic tools to the purification of crude synthetic oligomers: (1) pore size, (2) ligand chain length, and (3) gradient shape.

Figs. 1 and 2 show the chromatography of tritylated oligomers on $C_8$ stationary phases with pore diameters of both 300 and 80 Å. In general, oligomers greater than 25-mer are best purified on 300 Å packing, and those less than 25 are best purified on 80 Å packing. A mixture of two crude synthetic oligomers less than 25 bases in length were analyzed on 80 Å (Fig. 1A) and 300 Å (Fig. 1B) $C_8$ columns using the same mobile phases and gradients. The 16-mer and 17-mer differ only by a single thymidine at the 3' end, and the 16-mer would be a likely failure contaminant in the synthesis of the 17-mer.

Although both columns are able to resolve the two oligomers and separate them from synthesis by-products and detritylated failures, the 80 Å column shows improved resolution of the 16-mer from the 17-mer. The 80 Å column also shows improved resolution of the 16- and 17-mers from suspected depurinated-mers. The oligomers have increased retention times on the 80 Å column as compared to the 300 Å column. The improved resolution and increased retention times correlate with the increased surface area available on the 80 Å as compared to the 300 Å column.

In our experience on reversed-phase HPLC with triethylammonium phosphate (TEAP) (pH 7.0), purines as free bases, nucleotides and 5' mono- and diphosphates elute faster than pyrimidines (data not shown). Similar results have been reported by Ip *et al.*[10] using triethylammonium bicarbonate (TEAB) (pH 7.4).

The relative hydrophobic character of the exposed terminal base on the 3' end of the oligomers appears to influence the reversed phase elution. In this case the 16-mer containing the less hydrophobic pyrimidine elutes before the 17-mer containing the more hydrophobic purine.

A mixture of two crude synthetic oligomers longer than 25 bases were chromatographed on both the 80 Å (Fig. 2A) and 300 Å (Fig. 2B) $C_8$ columns. The 36- and 37-mers differ only by a single adenosine residue at the 5' end. Baseline resolution of the two oligomers is demonstrated on both columns, but the resolution of the 36- and 37-mers is favored on the 300 Å column.

The 300 Å packing also shows improved resolution of the suspected depurinated-mers from the products. A contaminant peak which co-elutes with the 37-mer on the 80 Å column is well resolved from the 37-mer on the 300 Å column. The more efficient purification of the 36- and 37-mers on the 300 Å column is the result of the increased ability of these oligomers to access the surface area within the pores on the 300 Å as compared to the 80 Å column. Most of the surface area available for the resolution of compounds is contained within the pores of the packing. The 36- and 37-mers, for example, have little access to the surface area within the 80 Å pores, while polymers less than 25-mer can be included in the pore volume.

The purification of tritylated oligomers on stationary phases having different ligand chain lengths are presented in Figs. 3 and 4. Oligomers less than 25 bases show little difference in resolution when run on the $C_8$ and $C_{18}$, 80 Å stationary phases.
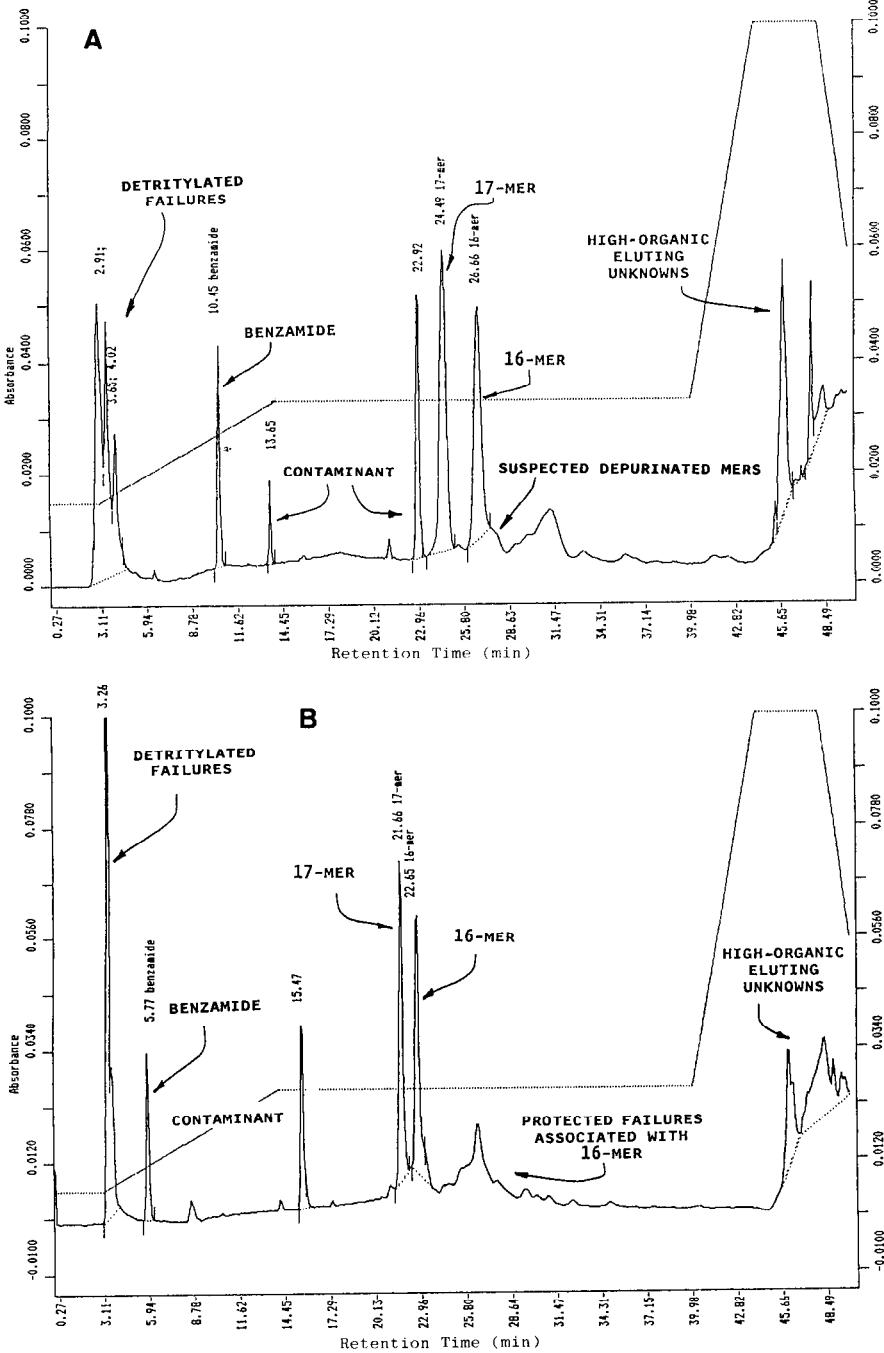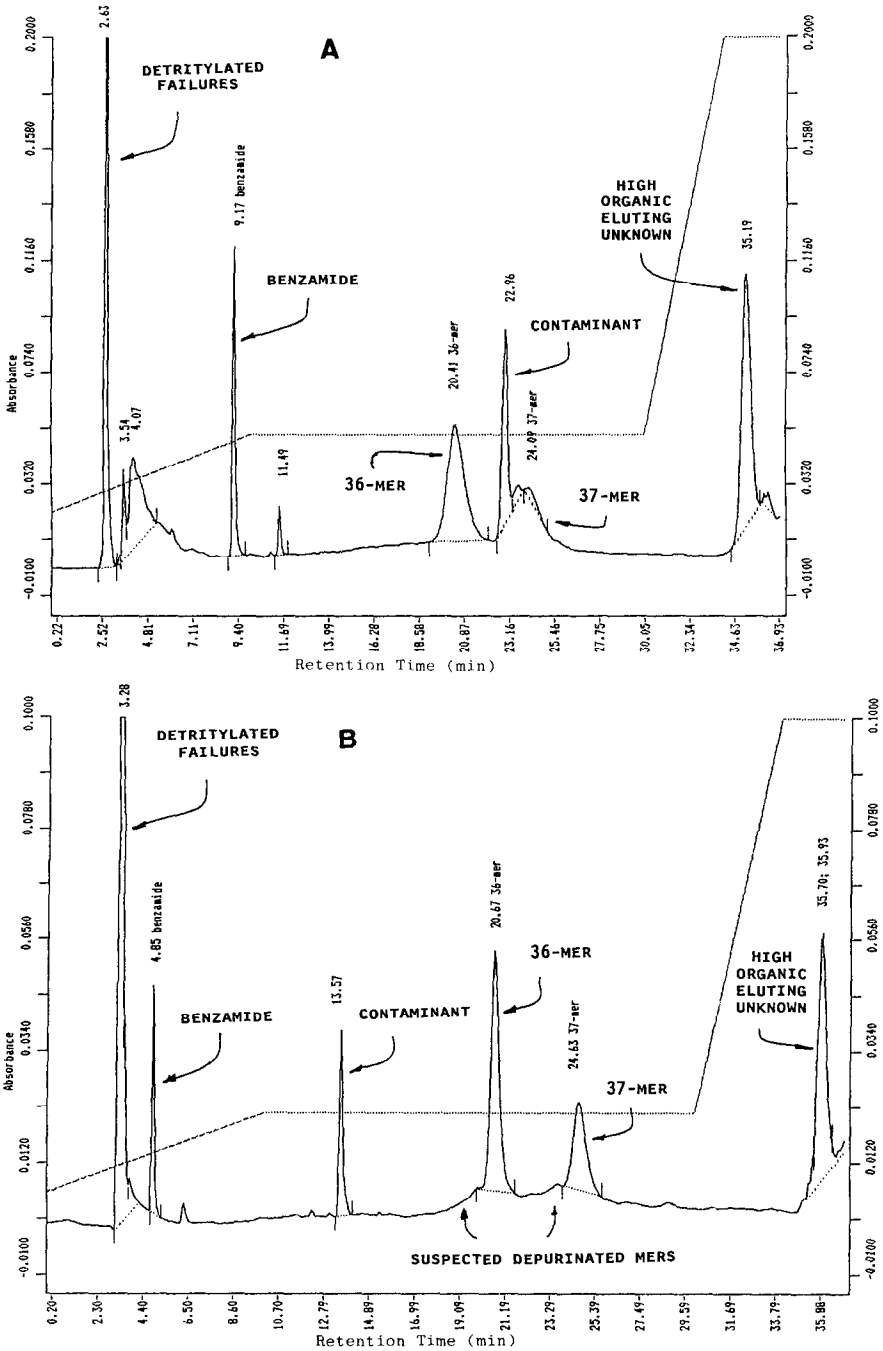
Fig. 1. Contribution of pore size to the separation of tritylated oligomers <25-mer. (A) Beckman Ultrasphere™ C$_8$, 80 Å (250 × 4.6 mm I.D.) column; (B) Beckman Ultrapore™ C$_8$, 300 Å (250 × 4.6 mm I.D.) column. Method 1.

Fig. 2. Contribution of pore size to the separation of tritylated oligomers >25-mer. (A) Beckman Ultrasphere $C_8$, 80 Å (250 × 4.6 mm I.D.) column; (B) Beckman Ultrapore $C_8$, 300 Å (250 × 4.6 mm I.D.) column. Method 2.

However, oligomers greater than 25-mer were better resolved on the $C_8$, 300 Å as compared to the $C_3$, 300 Å column.

A mixture of crude synthetic 16- and 17-mers were analyzed on both $C_8$ (Fig. 3A) and $C_{18}$ (Fig. 3B) 80 Å columns using identical mobile phases and gradients. Both $C_8$ and $C_{18}$ columns provided baseline resolution of the 16- and 17-mers in the isocratic portion of the gradient. The 16- and 17-mers are retained approximately one minute longer on the $C_{18}$ column and the peak widths are slightly larger than those on the $C_8$ column. In this case there is no real difference in resolution on $C_8$ and $C_{18}$ columns. However, a contaminating substance found in both the 16- and 17-mers is better resolved from the 17-mer on the $C_{18}$ column. The $C_{18}$ column, therefore, may have an advantage for the purification of product oligomers from closely eluting contaminants.

The purification of a mixture of crude synthetic oligomers greater than 25 bases is shown in Fig. 4. The 36- and 37-mers are being separated on both $C_3$ (Fig. 4A) and $C_8$ (Fig. 4B) 300 Å columns. The $C_8$ column shows significantly increased resolution of the 36- and 37-mers as compared to the $C_3$ column.

Gradient shape can be manipulated to control the balance between the degree of purification and the speed of the analysis required. The contribution of gradient shape to oligomer separation is illustrated in Figs. 5 and 6. All chromatography is carried out on a $C_8$, 300 Å column (75 × 4.6 mm I.D.) with identical mobile phases and variations in gradient shape. Fig. 5A shows the purification of the 36- and 37-mer with an initial gradient of 3% acetonitrile per min followed by an isocratic hold at 20% acetonitrile. With this gradient, the 36- and 37-mers are well resolved from one another, as well as from the contaminant, the synthesis by-products, and detritylated oligomers. In Fig. 5B, the gradient was changed to consist of a 1-min isocratic hold followed by a gradient of 17% acetonitrile per min. Under these stronger eluting conditions, the 36-, 37-mer and contaminating compound co-elute. However, the gradient permits the purification of the product oligomers from both the synthesis by-products and detritylated-mers in less than 5 min.

Similar gradients consisting of a 3-min isocratic hold followed by a 3.5% increase in acetonitrile content per min were used in Fig. 6 to purify a 76-mer and a 100-mer. The product oligomers are cleanly separated from the synthesis by-products and detritylated oligomers. Often oligomers for gene constructs or mutagenic studies are in the range of 20–80 bases or more in length. Purity requirements for these products may be very stringent. A single purification step may not be sufficient to provide product of the required purity[11].

*Detritylated oligomers*

The principles discussed for selection of stationary phase pore diameter hold for detritylated oligomers as well as tritylated oligomers. Oligomers > 25 in length are best analyzed on the wide-pore 300 Å packing, and oligomers < 25 are best run on 80 Å packing.

The separation of detritylated oligomers on stationary phases having different ligand chain lengths is presented in Figs. 7–9. Oligomers less than 25 bases were better resolved on $C_{18}$, 80 Å as compared to $C_8$, 80 Å columns. However, oligomers greater than 25 showed no difference in resolution when run on the $C_3$ and $C_8$, 300 Å phases.

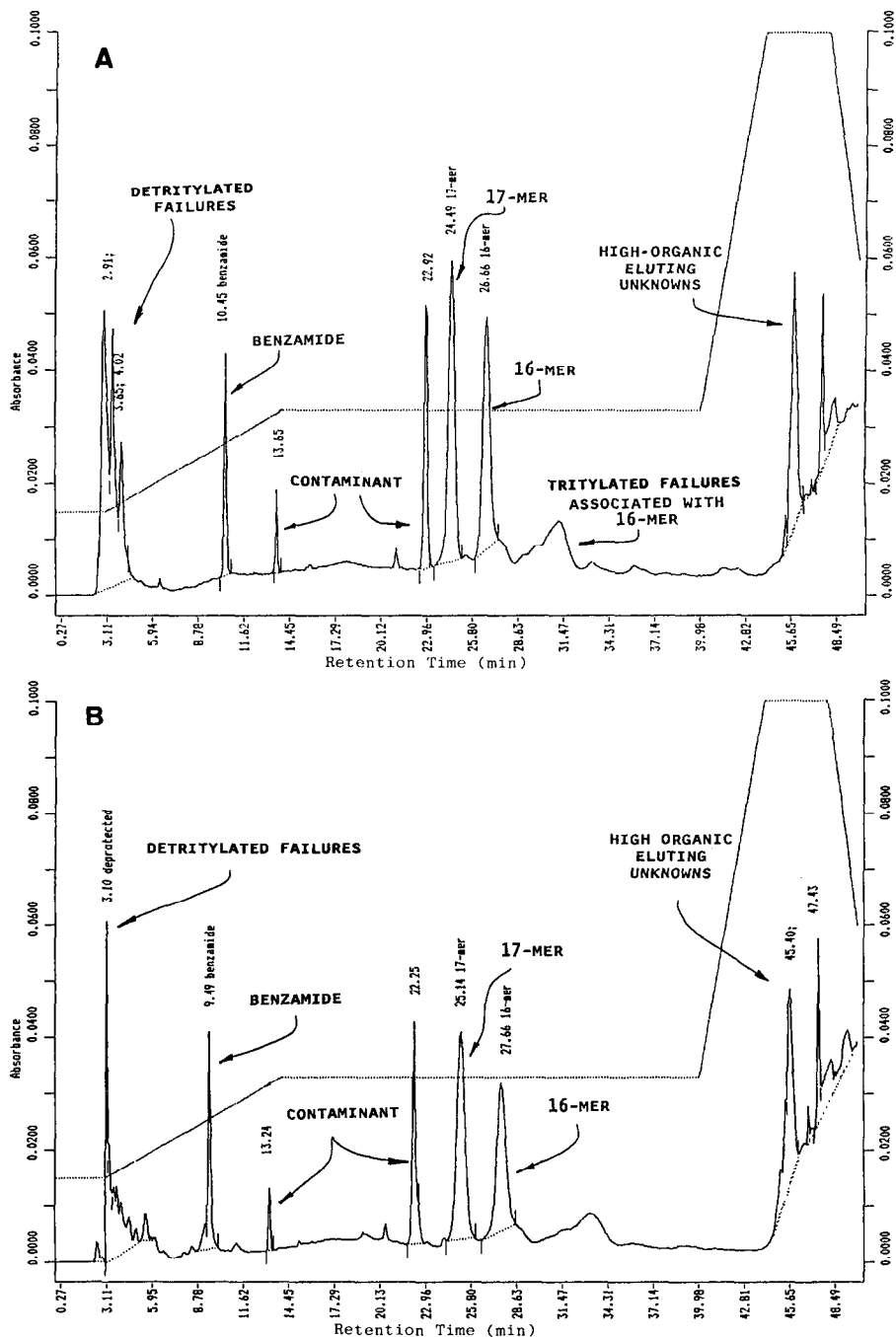Fig. 7A shows the separation of a mixture of two detritylated 17-mers on the

Fig. 3. Contribution of ligand chain length to the separation of tritylated oligomers <25-mer. (A) Beckman Ultrasphere $C_8$, 80 Å (250 × 4.6 mm I.D.) column; (B) Beckman Ultrasphere $C_{18}$, 80 Å (250 × 4.6 mm I.D.) column. Method 1.
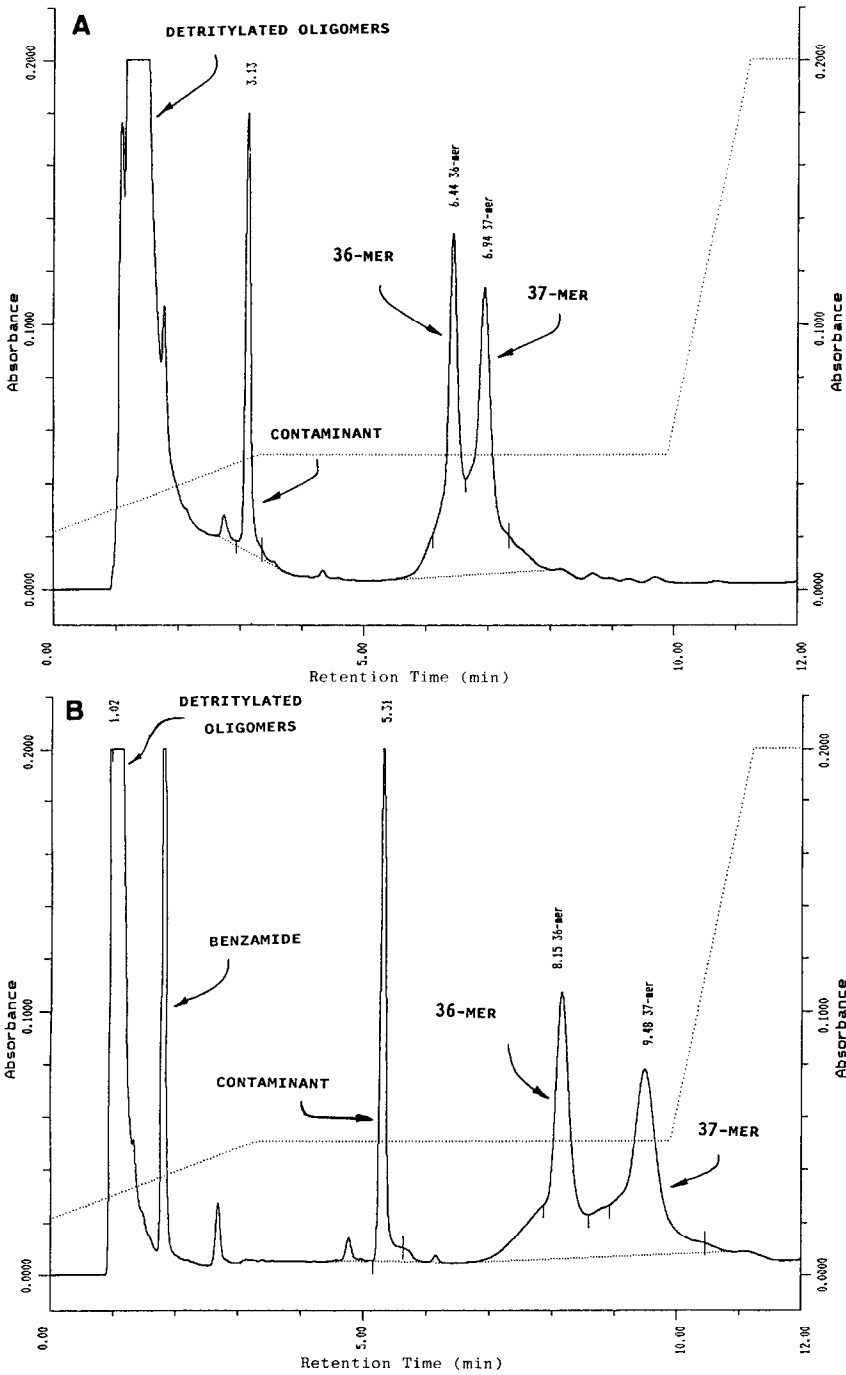
Fig. 4. Contribution of ligand chain length to the separation of tritylated oligomers >25-mer. (A) Beckman Ultrapore $C_3$ 300 Å (75 × 4.6 mm I.D.) column; (B) Beckman Ultrapore $C_8$, 300 Å (75 × 4.6 mm I.D.) column. Method 3.
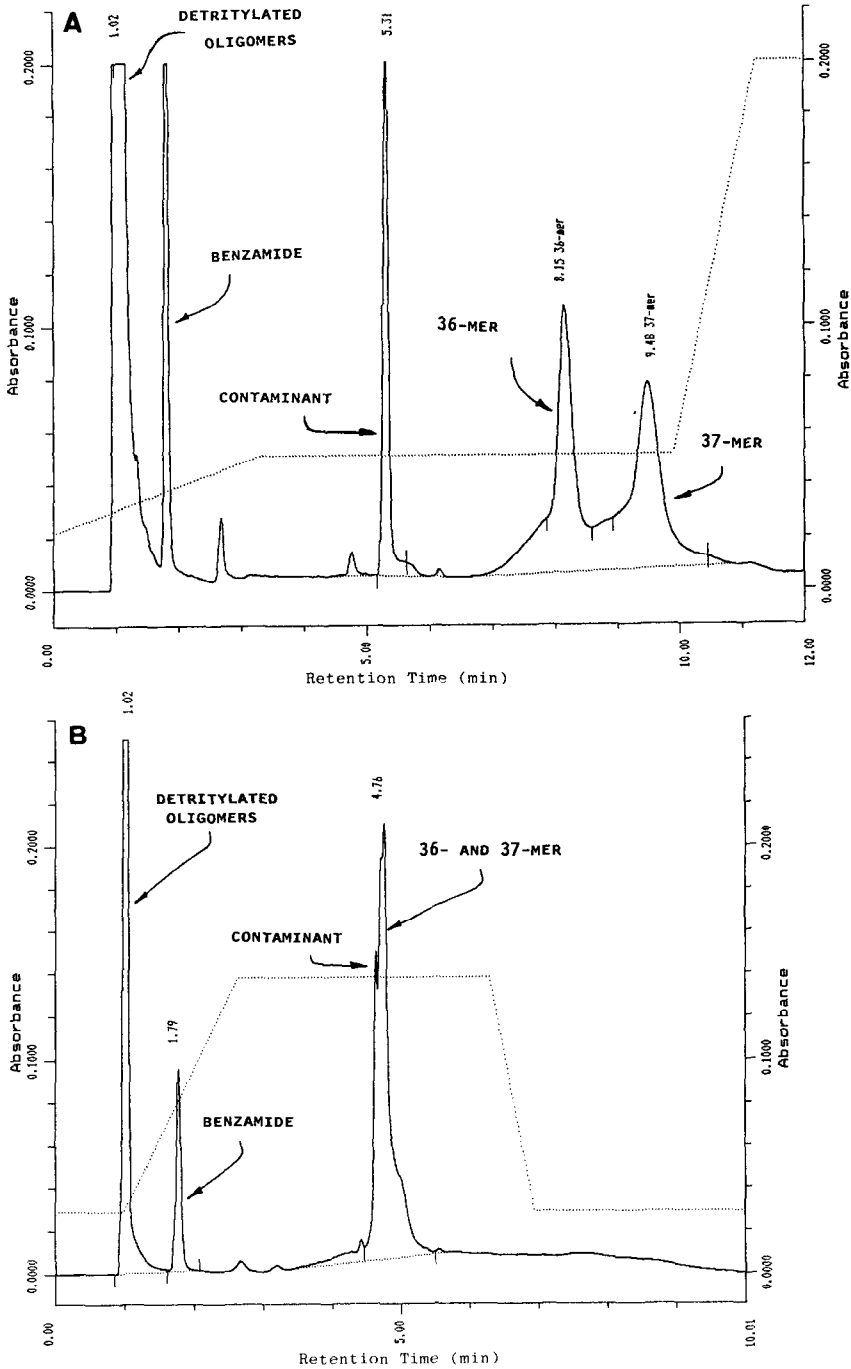
Fig. 5. Dependence of the separation of tritylated oligomers on the gradient shape. (A) Shallow gradient (method 3); (B) steeper gradient (method 4). Column: Beckman Ultrapore $C_8$, 300 Å (75 × 4.6 mm I.D.).
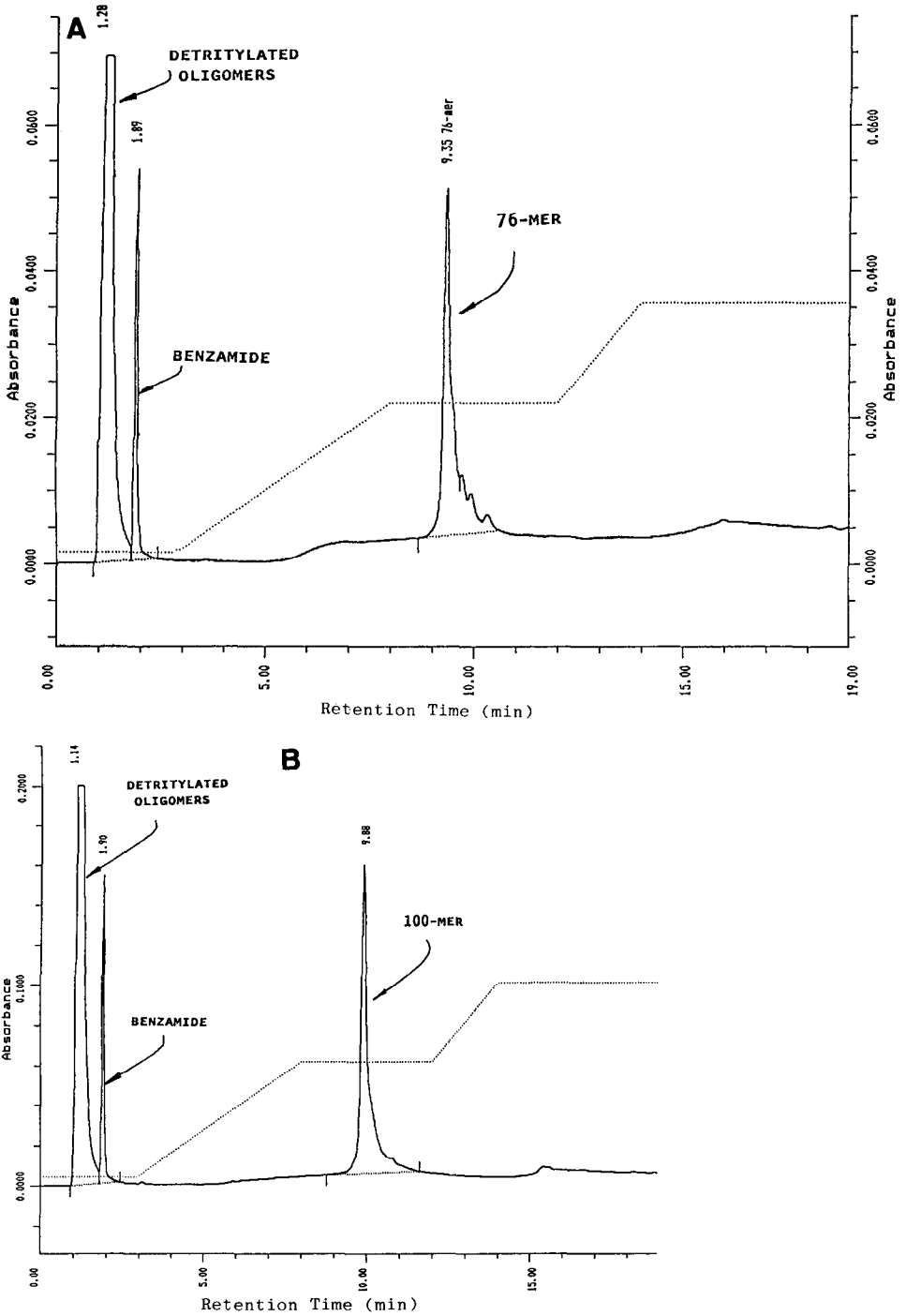
Fig. 6. Fast reversed-phase separation of tritylated oligomers >25-mer by gradient modification. (A) 76-mer; (B) 100-mer. Column: Beckman Ultrapore C$_8$, 300 Å (75 × 4.6 mm I.D.). Method 5.

$C_8$, 80 Å column. The two 17-mers have similar GC/AT ratios but very different sequences. The two oligomers are separated by 2.1 min on the $C_8$ column. Fig. 7B shows the same detritylated oligomers run on the $C_{18}$ column, with a separation of 2.5 min. The $C_{18}$ column provides improved separation of oligomers having similar base composition.

Oligomers greater than 25-mer were run on both $C_3$ and $C_8$, 300 Å columns using identical gradients and mobile phases. The 29- and 33-mer differ by both base sequence and composition. The GC/AT ratio for the 29-mer is 2.2 and for the 33-mer is 0.83. Fig. 8A shows the separation of the oligomers on the $C_3$, 300 Å column. The oligomers are separated by 0.62 min. Fig. 8B shows chromatography on the $C_8$, 300 Å column, and the separation is 0.51 min. The retention time is approximately 1 min longer on the $C_8$ column than on the $C_3$ column, but the increased retention time does not improve the resolution of the oligomers.

Gradient shape can be adjusted to further enrich detritylated oligomers. A shallower gradient offers increased resolution of similar composition oligomers, as well as a slight improvement in resolution from other compounds present in the reaction mixture. Fig. 9 illustrates two different gradients to separate oligomers less than 25-mer and Figs. 10 and 11 illustrate alteration of gradient shape for oligomers longer than 25.

Fig. 9A shows the separation of two 17-mers on a $C_{18}$ column with a 0.5%/min acetonitrile gradient. Fig. 9B shows the same oligomers on the same column with a 0.25%/min acetonitrile gradient. As the retention time increases from approximately 26 min to 48 min with the shallower gradient, the separation increases from 2.5 min to 4.3 min. In addition, the 17-mers appear to be better resolved from other compounds present in the reaction mixture.

Fig. 10 shows the chromatography of a 76-mer using a gradient of 0.5% acetonitrile per min. Fig. 11 contains chromatographs of the same oligomer using a 0.25%/min acetonitrile gradient. The separation of the oligomer from failures and reaction by-products is slightly improved by the slower gradient. As the length of the oligomer increases, the number of failure sequences increases, and the difficulty of purification also increases[12].

CONCLUSIONS

Convenient purification guidelines are offered for the reversed-phase HPLC of crude synthetic oligonucleotides:

(1) Tritylated oligomers up to 25-mer are best purified on $C_8$ or $C_{18}$ 80 Å columns. A combination of isocratic delays and shallow gradients with moderate strength mobile phases permits the simultaneous resolution of oligomers differing by as little as one base pair from suspected depurinated-mers as well as reaction by-products and detritylated-mers.

(2) Tritylated oligomers greater than 25-mer are best purified on 300 Å columns. The $C_8$ columns gave substantially increased purity as compared to the $C_3$ phase. Steeper gradients permitted oligomers up to 100-mer to be quickly separated from detritylated-mers and reaction by-products in as little as 10 min. A combination of shallower gradients and isocratic regions permitted the resolution of oligomers differing by as little as one base pair as well as the separation of depurinated-mers.
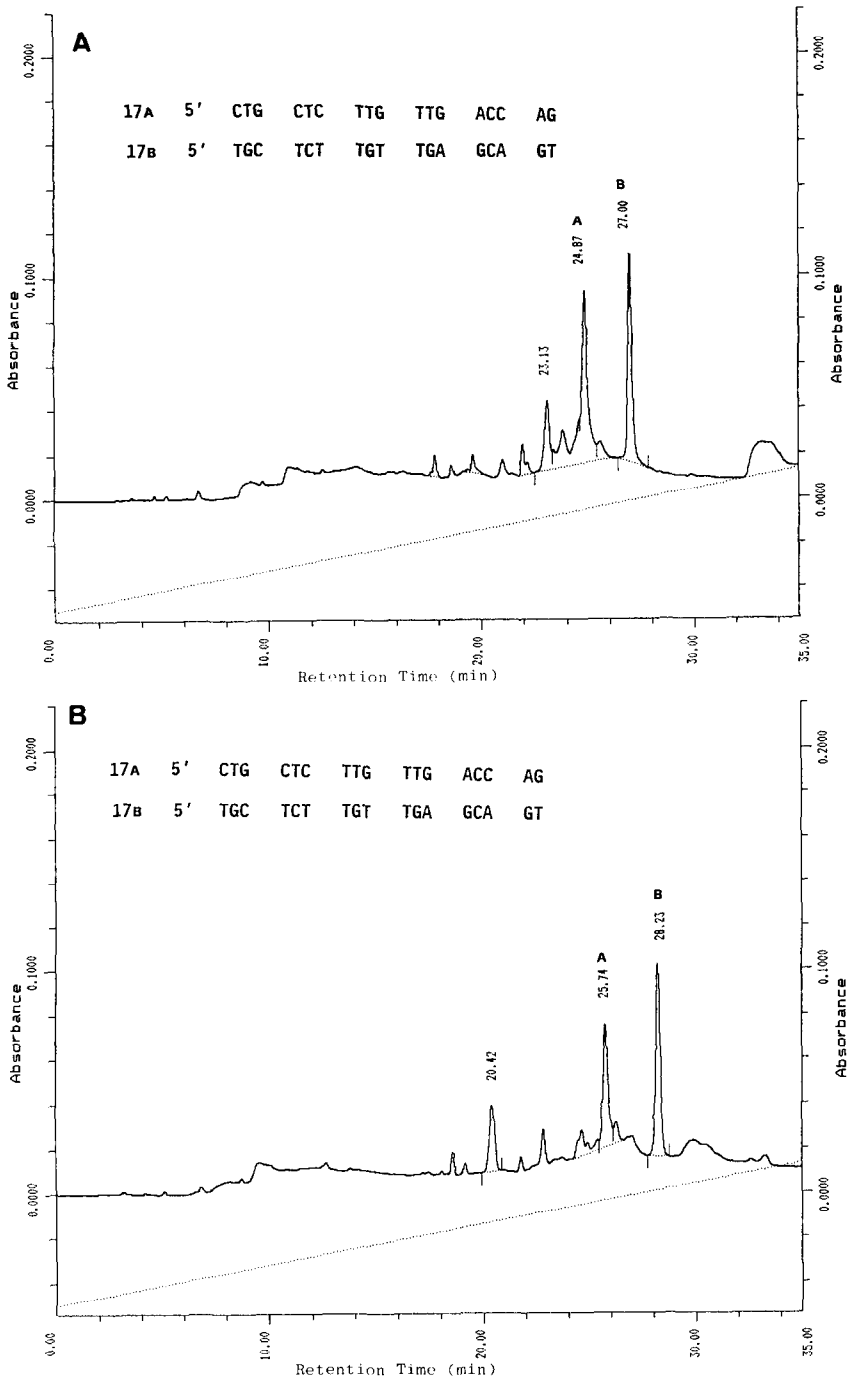
Fig. 7. Contribution of ligand chain length to the enrichment of detritylated oligomers <25-mer. (A) Beckman Ultrasphere $C_8$, 80 Å (250 × 4.6 mm I.D.) column; (B) Beckman Ultrasphere $C_{18}$, 80 Å (250 × 4.6 mm I.D.) column. Method 6.
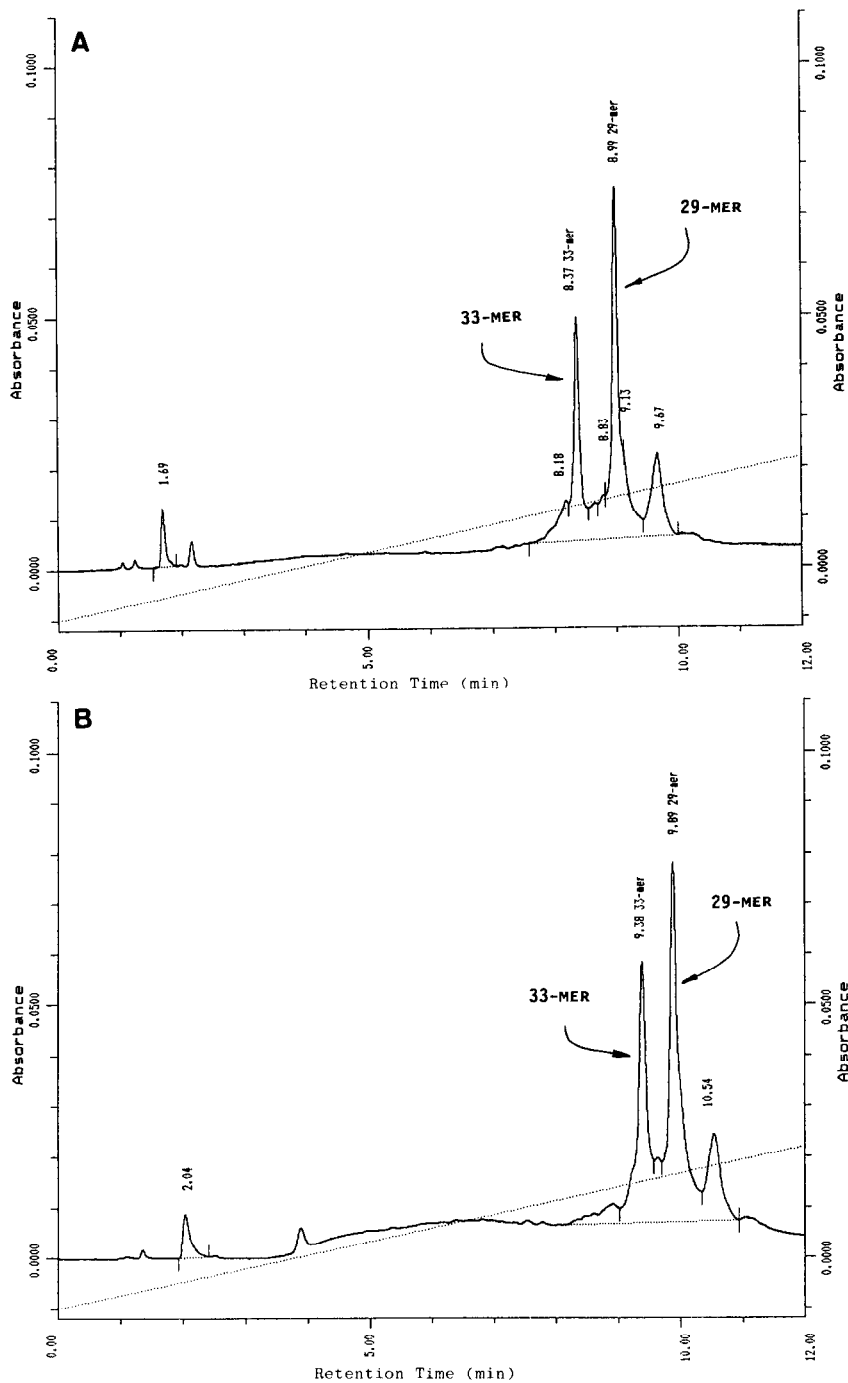
Fig. 8. Contribution of ligand chain length to the enrichment of detritylated oligomers >25-mer. (A) Beckman Ultrapore $C_3$, 300 Å (75 × 4.6 mm I.D.) column; (B) Beckman Ultrapore $C_8$, 300 Å (75 × 4.6 mm I.D.) column. Method 7.
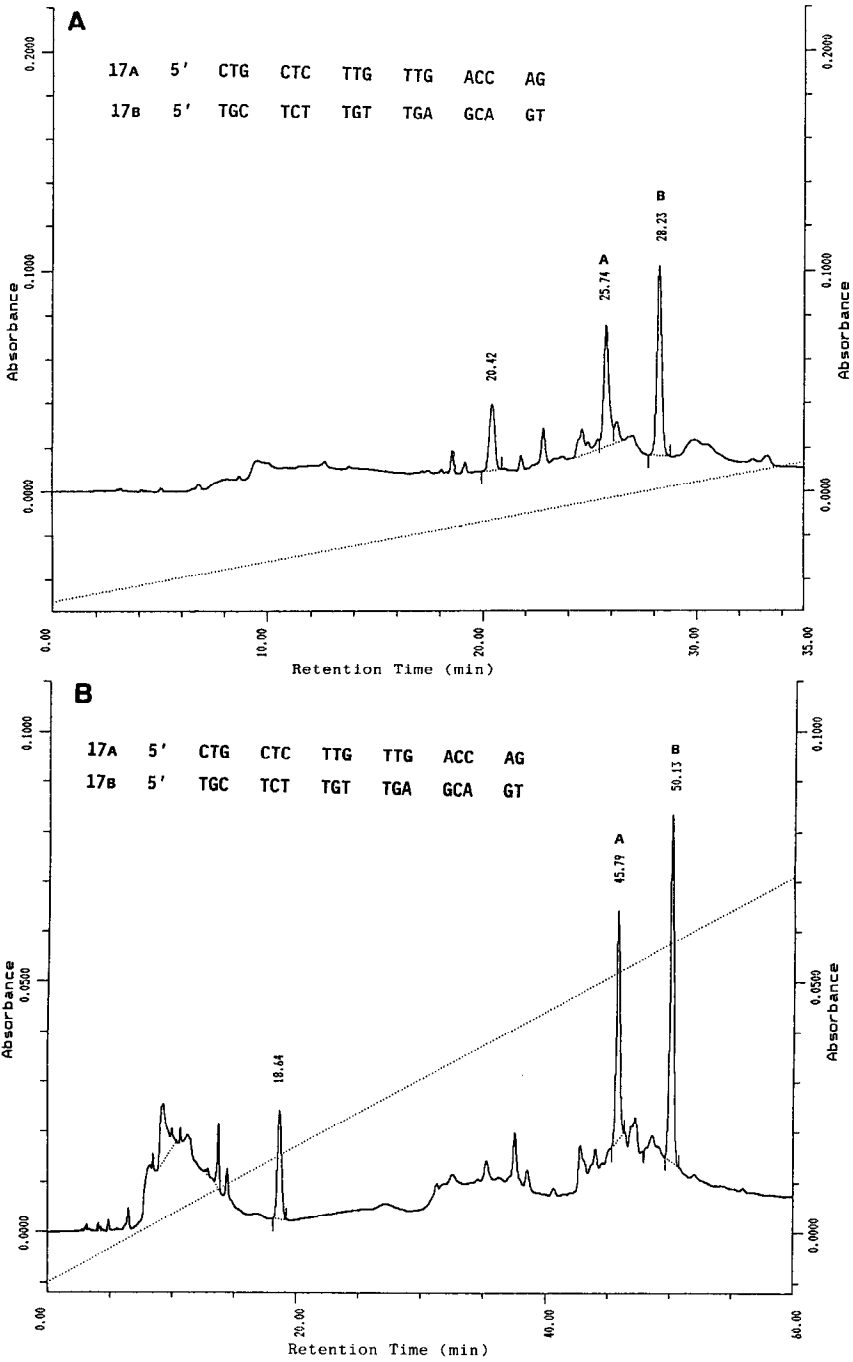
Fig. 9. Dependence of the enrichment of detrytylated oligomers on gradient shape. (A) 0.5% acetonitrile per min, method 6; (B) 0.25% acetonitrile per min, method 8. Column: Beckman Ultrasphere $C_{18}$, 80 Å (250 × 4.6 mm I.D.).
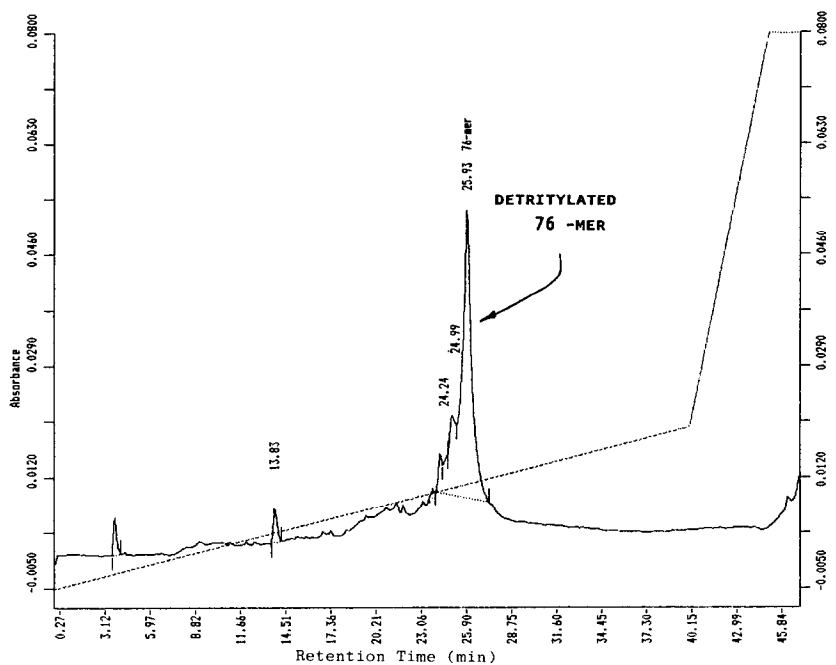
Fig. 10. Chromatography of a 76-mer with 0.5%/min actonitrile gradient. Column: Beckman Ultrapore $C_8$, 300 Å (250 × 4.6 mm I.D.). Method 6.
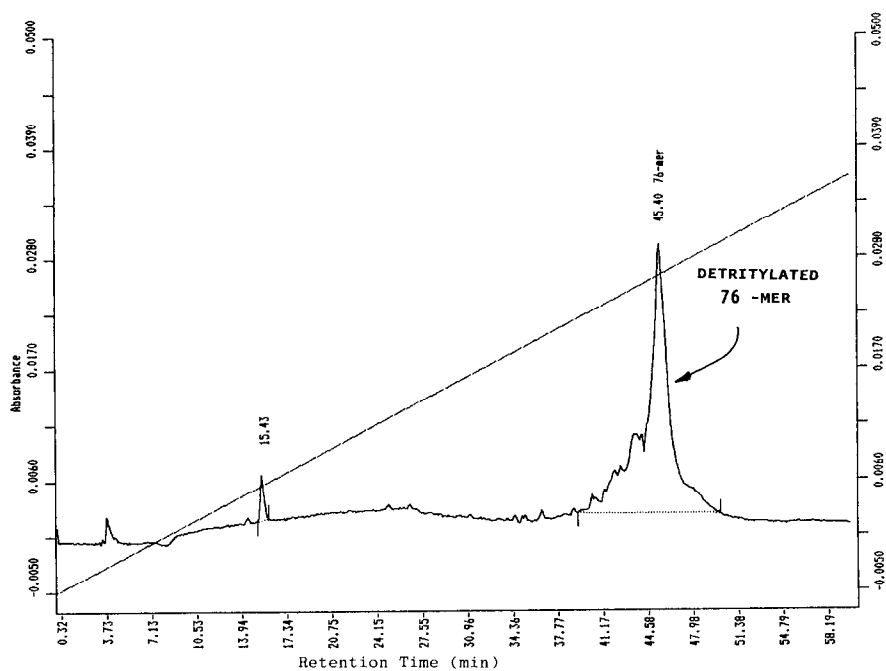


Fig. 11. Chromatography of a 76-mer with 0.25%/min acetonitrile gradient. Column: Beckman Ultrapore $C_8$, 300 Å (250 × 4.6 mm I.D.). Method 8.

(3) Detritylated oligomers require a modified approach. Up to 25-mer, they are best purified on $C_{18}$, 80 Å columns with much lower organic concentrations and shallower gradients than used for tritylated oligomers. Similar oligomers differing by base composition can also be separated under these conditions.

(4) Using methods described for shorter detritylated oligomers, 25- to 100-mer polymers can be enriched on both $C_3$ and $C_8$, 300 Å columns. Longer, shallower gradients will increase the purity of the collected oligomer. Chromatography of detritylated oligomers is best used for enrichment rather than for true purification.

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