Journal of Chromatography, 447 (1988) 212–220
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 549

Note

Separation of oligonucleotides by high-performance ion-exchange chromatography on a non-porous ion exchanger

YOSHIO KATO*, TAKASHI KITAMURA, AKANE MITSUI, YOSUKE YAMASAKI and TSUTOMU HASHIMOTO
Central Research Laboratory, Tosoh Corporation, Tonda, Shinnanyo, Yamaguchi 746 (Japan)
and
TOMOAKI MUROTSU, SHINICHI FUKUSHIGE and KENICHI MATSUBARA
Institute for Molecular and Cellular Biology, Osaka University, Yamadaoka, Suita, Osaka 565 (Japan)
(Received February 2nd, 1988)

Oligonucleotides are of increasing importance in the field of gene technology, and the requirement for highly pure oligonucleotides is increasing. They can be prepared rather easily in a short time by using an automated synthesizer. However, the samples synthesized usually contain many impurities together with the target oligonucleotides, and hence purifications are inevitable. Although polyacrylamide gel electrophoresis and conventional liquid chromatography are mainly employed for this purpose, high-performance liquid chromatography (HPLC), in particular reversed-phase and ion-exchange chromatography, has often been adopted[1–25]. However, the resolution or separation time in HPLC has not been fully satisfied, especially for large oligonucleotides and improvement is required.

Recently, we demonstrated that ion-exchange chromatography on non-porous ion exchangers is very effective for the rapid separation of proteins with high resolution[26]. We have now investigated the separation of oligonucleotides on a non-porous anion exchanger.

EXPERIMENTAL

Chromatographic measurements were performed with a system consisting of a double plunger pump, Model CCPM, and a variable-wavelength UV detector, Model UV-8000, operated at 260 nm (Tosoh, Tokyo, Japan). The column was TSKgel DEAE-NPR (35 mm × 4.6 mm I.D.) (Tosoh) packed with non-porous spherical hydrophilic resins of 2.5 μm in diameter whose surfaces are chemically bonded with diethylaminoethyl groups[26]. Elution was usually performed with a linear gradient of sodium chloride in 20 m*M* Tris–HCl buffer (pH 9.0) or 1,3-diaminopropane–HCl buffer (pH 10.5) at a flow-rate of 1.5 ml/min and 25°C. In some separations, however, the conditions were varied to study their effects.

All oligonucleotide samples were from Pharmacia (Uppsala, Sweden), except three linker oligonucleotides, d(GCGATCGC), d(CGAGCTCG) and d(GCAGCTGC), and a crude sample of a pentadecanucleotide, d(AACGCACAC-

0021-9673/88/$03.50

TAAACG). The linker oligonucleotides were from Wako (Osaka, Japan). The crude sample of pentadecanucleotide was a gift from Mr. Ikari of our laboratory and the protecting groups were removed after synthesis.

RESULTS AND DISCUSSION

Fig. 1 shows a separation of a mixture of oligoadenylic acids with chain lengths of 1, 2, 3, 4, 5, 8, 10, 12, 16 and 20 nucleotides. These comparatively small oligonucleotides could be separated well in as short a time as 8 min. Oligonucleotides were eluted as very sharp peaks. However, mononucleotide was eluted as a slightly broad tailing peak. This is probably because the packing material of DEAE-NPR is resin-based and is supposed to have very small pores, although it is said to be non-porous. If small molecules like mononucleotide enter such very small pores, the diffusion rate there should be slow, which results in broad and tailing peaks.

Fig. 2. shows a separation of a hydrolysate of polyadenylic acid containing from the 17-mer through approximately the 100-mer. Baseline separations were obtained for up to the 32-mer and peaks appeared for up to about the 70-mer, and yet the separation was completed in less than 20 min. Although a similar separation to that in Fig. 2 has been reported by others, the separation time was much longer than 20 min, about 20 h[12]. As far as we know, this is the first rapid separation of large oligonucleotides with high resolution.

Fig. 3 shows a separation of four tetramers of adenylic, cytidylic, thymidylic and guanylic acids. The four components were separated well in a short time, indicating that ion-exchange chromatography on a non-porous anion exchanger is also very useful to separate oligonucleotides according to base composition. However, it was not so effective to separate sequence isomers. The separation of a mixture of

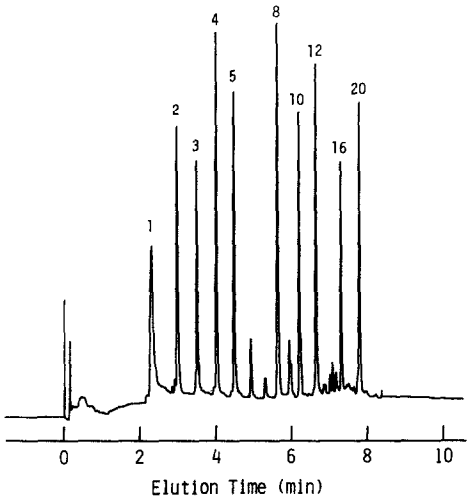

Fig. 1. Chromatogram of a mixture of oligoadenylic acids with chain lengths of 1, 2, 3, 4, 5, 8, 10, 12, 16 and 20 nucleotides (0.1 μg each). The separation was performed on a TSKgel DEAE-NPR column with a 20-min linear gradient from 0 to 1 *M* sodium chloride in 20 m*M* Tris–HCl buffer (pH 9.0) at a flow-rate of 1.5 ml/min and 25°C.

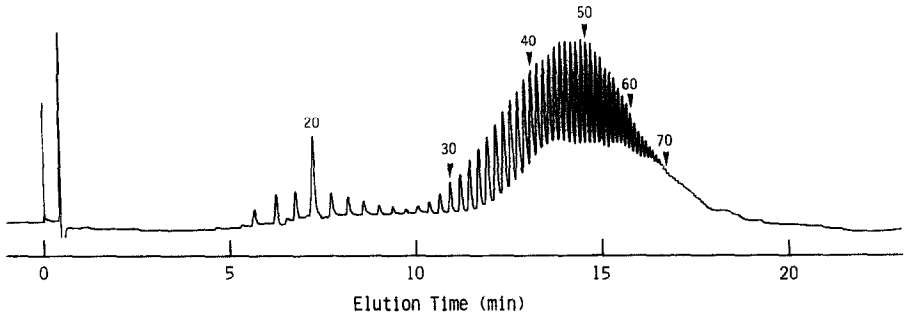

Fig. 2. Chromatogram of a hydrolysate of polyadenylic acid containing from the 17-mer through approximately the 100-mer. The separation was performed on two TSKgel DEAE-NPR columns connected in series with a 60-min linear gradient from 0.25 to 1 *M* sodium chloride in 20 m*M* Tris–HCl buffer (pH 9.0) at a flow-rate of 1.0 ml/min and 25°C.

d(GCGATCGC), d(CGAGCTCG) and d(GCAGCTGC) was examined under the same conditions as in Fig. 3. d(GCGATCGC) and d(CGAGCTGC) were eluted as a single peak although they were almost completely separated from d(CGAGCTCG).

Fig. 4 shows a separation of a crude sample of synthetic pentadecamer. The largest peak is an objective 15-mer and it was completely separated from contaminants in about 5 min. Purification and purity analysis of synthetic oligonucleotides are inevitable and very important in the syntheses of DNA or RNA. For these purposes, ion-exchange chromatography on a non-porous anion exchanger is very useful.

Fig. 5 shows the loading capacity in ion-exchange chromatography of oligonucleotides on a non-porous anion exchanger. A pure sample of the 16-mer of adenylic acid was separated using various sample loads, and the peak width was plotted

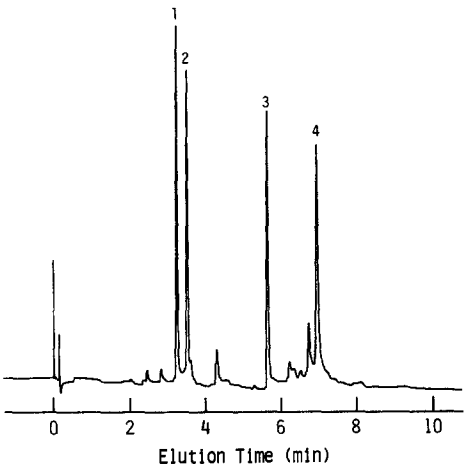

Fig. 3. Chromatogram of a mixture of tetramers of adenylic acid (1, 0.1 μg), cytidylic acid (2, 0.2 μg), thymidylic acid (3, 0.1 μg) and guanylic acid (4, 0.4 μg). The separation was performed on a TSKgel DEAE-NPR column with a 20-min linear gradient from 0 to 1 *M* sodium chloride in 20 m*M* 1,3-diaminopropane–HCl buffer (pH 10.5) at a flow-rate of 1.5 ml/min and 25°C.

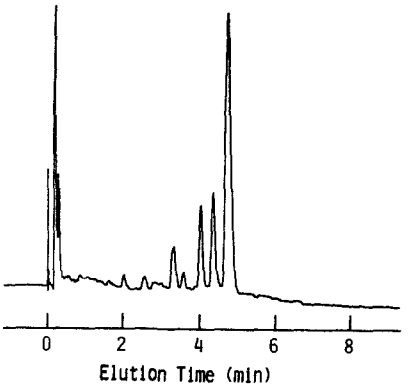

Fig. 4. Chromatogram of a crude sample of synthetic 15-mer, d(AACGCACACTAAACG). The separation was performed on a TSKgel DEAE-NPR column with a 60-min linear gradient from 0 to 1 *M* sodium chloride in 20 m*M* 1,3-diaminopropane–HCl buffer (pH 10.5) at a flow-rate of 1.5 ml/min and 25°C.

against the sample load. The peak width was constant at sample loads up to 0.2 μg, and then became broader with further increase in the sample load. Therefore, the maximum sample load in order to obtain the highest resolution is rather small, about 0.2 μg, for pure samples although it is expected to be much larger for samples containing many components, as in the separation of proteins[26]. This low loading capacity must be due to the small surface area of DEAE-NPR and it is the biggest disadvantage of non-porous packing materials.

The recovery of oligonucleotides from a TSKgel DEAE-NPR column is shown in Table I. A pure sample of the 16-mer of adenylic acid and a mixture of oligoadenylic acids with chain lengths of 12–18 nucleotides were separated at pH 9.0, and the recovery was estimated from the areas of the peaks eluted. As controls, we used peak areas observed when the column was replaced with an empty 1 mm I.D. stainless-steel tube of 1 ml total inner volume. Both samples were recovered in high yield,

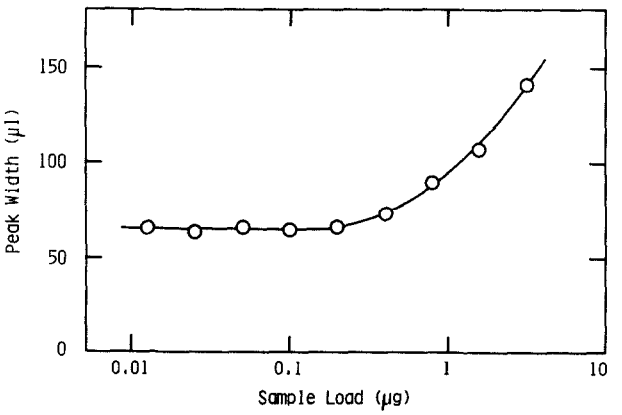

Fig. 5. Dependence of the peak width on sample load in the separation of the 16-mer of adenylic acid on TSKgel DEAE-NPR. Elution conditions as in Fig. 1.

TABLE I

RECOVERY OF OLIGONUCLEOTIDES FROM A TSKgel DEAE-NPR COLUMN

Samples were separated under the conditions in Fig. 1.

Sample	*Recovery (%)*
Hexadecaadenylic acid (0.1 μg)	93
A mixture of oligoadenylic acids with chain lengths of 12–18 nucleotides (0.5 μg)	96

more than 90%. Therefore, high recoveries are expected even with low sample loads such as 0.1 and 0.5 μg. However, the chromatographic conditions, especially the eluent pH, is very important to achieve high recovery, as described later.

The effects of some chromatographic conditions were studied. First, the eluent pH was examined. When oligoadenylic acids were separated at pH 8.5–10.5, similar separations as in Figs. 1 and 2 were obtained. However, the peaks were subject to tailing and the recovery decreased at pH 4.5–7.5, especially for large molecules. Therefore, the eluent pH should be $\geqslant$ 8.5 for the separation of oligonucleotides. Furthermore, a mixture of decamers of adenylic and thymidylic acids was eluted as a broad peak at pH 4.5–9.5, while the two components were eluted separately as sharp peaks at pH 10.5. Similar results were also obtained for a mixture of 16-mer of adenylic and thymidylic acids although the trimers were eluted separately over the whole pH range studied (4.5–10.5). Consequently, the eluent pH must be high such as 10.5 for the separation of samples containing complementary components. When a mixture of four tetramers of adenylic, cytidylic, thymidylic and guanylic acids was separated, comparatively similar results were obtained at pH 8.5–9.5, while the four components were eluted over a wider range of elution time at pH 10.5 than at lower pH; compare Fig. 6 with Fig. 3. Accordingly, the range pH 8.5–9.5 is better for a

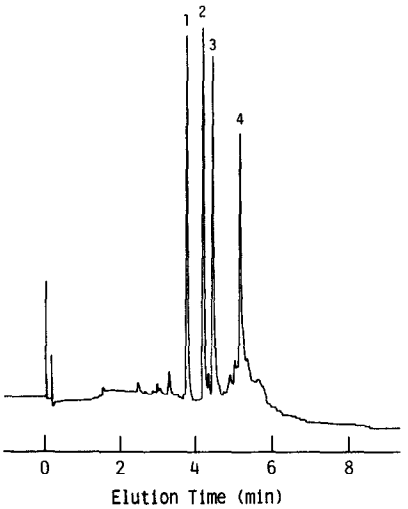

Fig. 6. Chromatogram of a mixture of four tetramers. The same mixture as in Fig. 3 was separated under the same conditions as in Fig. 3, except that the buffer was 20 m*M* Tris–HCl (pH 9.0).

separation based on chain length, while pH 10.5 is better for a separation based on base composition. There is no problem in operating TSKgel DEAE-NPR at high pH because it is chemically very stable. This is one of its advantages over silica-based packing materials.

Next, the effect of the type of salt was examined. Sodium chloride and sodium perchlorate were compared. Similar separations were obtained for mixtures of oligoadenylic acids except very small ones (lower than the trimer), although sodium perchlorate was approximately 2.5 times more effective than sodium chloride in the elution of oligonucleotides, compare Fig. 7 with Fig. 1. Therefore, it seems that the type of salt has little effect on the separation of homooligonucleotides provided that the gradient steepness is properly adjusted, although sodium perchlorate may be better than sodium chloride for the separation of very small oligonucleotides (lower than the trimer). However, four tetramers of adenylic, cytidylic, thymidylic and guanylic acids were eluted very closely when sodium perchlorate was used, as is seen from a comparison of Figs. 6 and 8. Consequently, sodium perchlorate seems better than sodium chloride for a separation based on chain length only. Conversely sodium chloride is better than sodium perchlorate for a separation based on base composition.

The effect of addition of organic solvent to the eluent was very small, although oligonucleotides were eluted slightly earlier and four tetramers of adenylic, cytidylic, thymidylic and guanylic acids were eluted in a slightly narrower range of elution time with the addition of 10–30% acetonitrile.

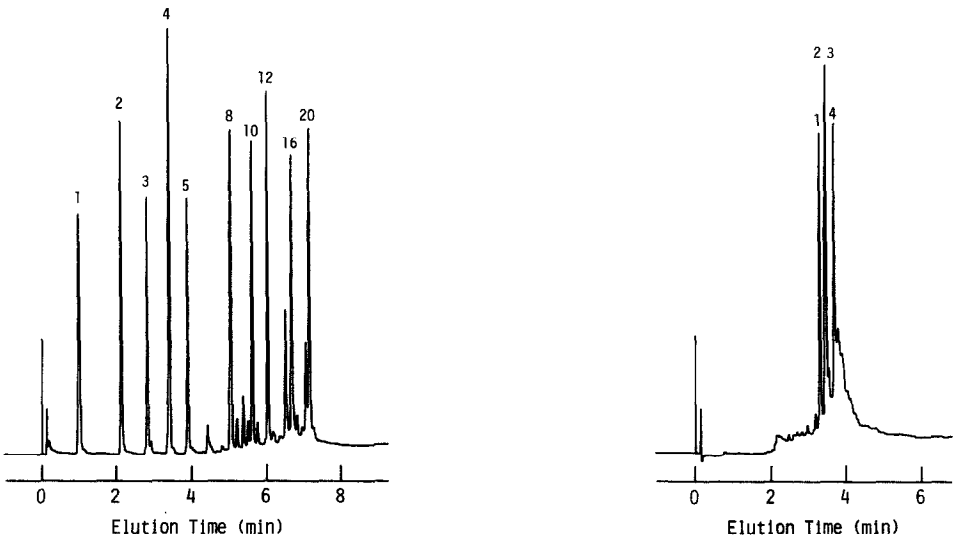

Fig. 7. Chromatogram of a mixture of oligoadenylic acids. The same mixture as in Fig. 1 was separated under the same conditions as in Fig. 1, except that the elution was performed with a 20-min linear gradient from 0 to 0.4 *M* sodium perchlorate in 20 m*M* Tris–$HClO_4$ buffer (pH 9.0).

Fig. 8. Chromatogram of a mixture of four tetramers. The same mixture as in Fig. 6 was separated under the same conditions as in Fig. 6, except that the elution was performed with a 20-min linear gradient from 0 to 0.4 *M* sodium perchlorate in 20 m*M* Tris–$HClO_4$ buffer (pH 9.0).

The effect of temperature was examined by separating oligonucloetides at 25–65°C. With increasing temperature, the elution of oligonucleotides was slightly delayed and the recovery tended to decrease gradually, especially above 45°C. The resolution was almost constant in the range of 25–45°C, and gradually decreased at higher temperature. Accordingly, a temperature of around 25°C seems appropriate.

Fig. 9 shows the effect of gradient steepness on resolution. A mixture of oligoadenylic acids was separated with a linear gradient from 0 to 1 *M* sodium chloride at a flow-rate of 1.5 ml/min. The gradient time was varied from 5 to 80 min. The resolution increased continuously with increasing gradient time. However, the dependence of resolution on gradient time became less pronounced at times longer than about 20 min. Because longer gradient times result in longer separation times and more dilution of the sample during separation, gradient times of around 20 min, which correspond to a gradient steepness of about 50 m*M* sodium chloride/min, seem to be a good compromise in general. However, higher resolution can be attained by adopting a shallower salt gradient.

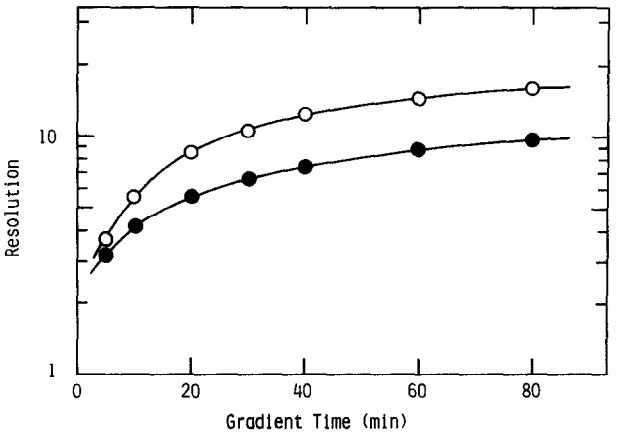

Fig. 9. Dependence of the resolution on gradient time in the separation of oligonucleotides on a TSKgel DEAE-NPR column. The same mixture of oligoadenylic acids as in Fig. 1 was separated under the conditions in Fig. 1 except that the gradient time was varied between 5 and 80 min. Resolutions were calculated for a pair of tetramer and pentamer (●) and a pair of 16-mer and 20-mer (○) from their peak widths and elution positions.

Fig. 10 shows the effect of flow-rate on resolution. A mixture of oligoadenylic acids was separated with a 20-min linear gradient from 0 to 1 *M* sodium chloride at flow-rates of 0.25–2.0 ml/min. The resolution increased with increasing flow-rate. However, the effect of flow-rate became small at rates above 1 ml/min, and especially for the pair of small oligonucleotides the resolution was almost constant at flow-rates between 1 and 2 ml/min. Because higher flow-rates result in higher column back pressure, rates of 1.0–1.5 ml/min seem to be a good compromise.

The effect of column length was also examined. Oligoadenylic acids were separated on one- and two-column systems. When the separation was performed with a gradient of 50 m*M* sodium chloride/min, almost identical separations were ob-

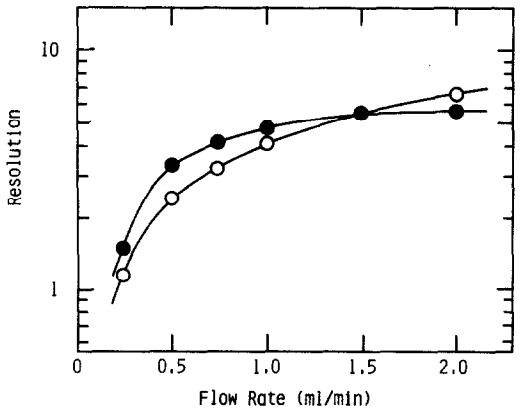

Fig. 10. Dependence of the resolution on flow-rate in the separation of oligonucleotides on a TSKgel DEAE-NPR column. The same mixture of oligoadenylic acids as in Fig. 1 was separated under the conditions in Fig. 1 except that the flow-rate was varied between 0.25 and 2.0 ml/min. Resolutions were calculated for a pair of tetramer and pentamer (●) and a pair of decamer and dodecamer (○) from their peak widths and elution positions.

tained, compare Figs. 11 and 1), suggesting that the column length has little influence on the separation when the gradient of sodium chloride is steep. With a gradient of 12.5 m*M* sodium chloride/min, however, a better separation was obtained on the two-column system than on the one-column system, compare Figs. 12 and 2). Therefore, it seems that higher resolution can be attained by using longer columns in the case of a shallow salt gradient.

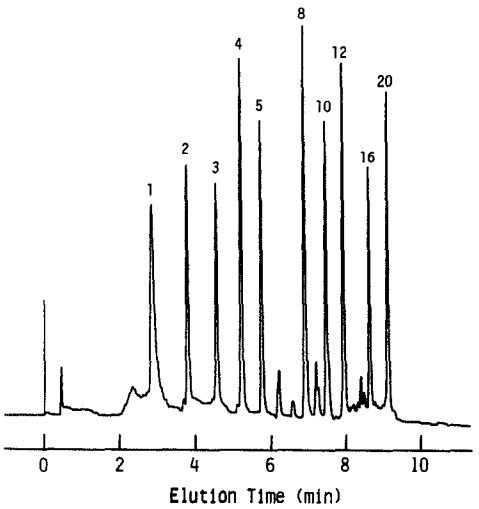

Fig. 11. Chromatogram of a mixture of oligoadenylic acids. The same mixture as in Fig. 1 was separated under the same conditions as in Fig. 1 except that two TSKgel DEAE-NPR columns were used.

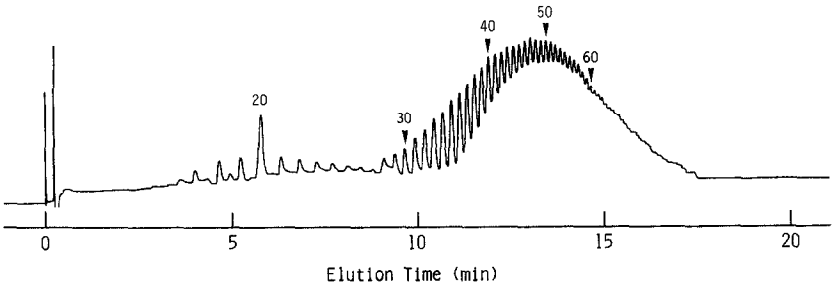

Fig. 12. Chromatogram of an hydrolysate of polyadenylic acid. The same mixture as in Fig. 2 was separated under the same conditions as in Fig. 2 except that one TSKgel DEAE-NPR column was used.

As demonstrated above, the non-porous anion exchanger, TSKgel DEAE-NPR, is very useful to separate oligonucleotides. Oligonucleotides can be separated rapidly with very high resolution and recovery, mainly based on the chain length at pH 8.5–9.5. The separation of oligonucleotides mainly according to the base composition is also possible by using an eluent of high pH around 10.5. Chromatographic conditions such as the eluent pH, type of salt, addition of organic solvent to the eluent, temperature, gradient steepness, flow-rate and column length influence the retention, selectivity, recovery, resolution, etc.

REFERENCES

1 J. F. Burd, J E. Larson and R. D. Wells, *J. Biol. Chem.*, 250 (1975) 6002.
2 G. C. Walker, O C. Uhlenbeck, E. Bedows and R. I. Gumport, *Proc. Natl. Acad. Sci. U.S.A.*, 72 (1975) 122.
3 J. B. Dodgson and R. D. Wells, *Biochemistry*, 16 (1977) 2367.
4 H.-J. Fritz, R. Belagaje, E. L. Brown, R. H. Fritz, R. A. Jones, R. G. Lees and H. G. Khorana, *Biochemistry*, 17 (1978) 1257.
5 D. Molko, R. Derbyshire, A. Guy, A. Roget, R. Teoule and A. Boucherle, *J. Chromatogr.*, 206 (1981) 493.
6 P. J. Cayley, R. E. Brown and I. M. Kerr, *J. Liq. Chromatogr.*, 5 (1982) 2027.
7 L W. McLaughlin and E. Graeser, *J. Liq. Chromatogr.*, 5 (1982) 2061.
8 L. W. McLaughlin and E. Romaniuk, *Anal. Biochem.*, 124 (1982) 37.
9 J. D. Pearson and F. E. Regnier, *J. Chromatogr.*, 255 (1983) 137.
10 W. Haupt and A. Pingoud, *J. Chromatogr.*, 260 (1983) 419.
11 R. Bischoff and L. W. McLaughlin, *J. Chromatogr.*, 270 (1983) 117.
12 R. Bischoff and L. W. McLaughlin, *J. Chromatogr.*, 296 (1984) 329.
13 C. R. Newton, A. R. Greene, G. R. Heathcliffe, T. C. Atkinson, D. Holland, A. F. Markham and M. D. Edge, *Anal. Biochem.*, 192 (1983) 22.
14 M. Colpan and D. Riesner, *J. Chromatogr.*, 296 (1984) 339.
15 H. Schott and H. Eckstein, *J. Chromatogr.*, 296 (1984) 363.
16 M. Cubellis, G. Marino, L. Mayol, G. Piccialli and G. Sannia, *J. Chromatogr.*, 329 (1985) 406.
17 D. J. Anderson, A. J. Taylor and R. J. Reischer, *J. Chromatogr.*, 350 (1985) 313.
18 T. Middleton, W. C. Herlihy, P. R. Schimmel and H N. Munro, *Anal. Biochem.*, 144 (1985) 110.
19 R. R. Drager and F. E. Regnier, *Anal. Biochem.*, 145 (1985) 47.
20 T. R. Floyd, L. W. Yu and R. A. Hartwick, *Chromatographia*, 21 (1986) 402.
21 K. Ashman, A. Bosserhoff and R. Frank, *J. Chromatogr.*, 397 (1987) 137.
22 H. Seliger and G. Schmidt, *J. Chromatogr.*, 397 (1987) 141.
23 K. Makino, H. Ozaki, T. Matsumoto, H. Imaishi, T. Takeuchi and T. Fukui, *J. Chromatogr.*, 400 (1987) 271.
24 U. Birsner, U. Gilles, P. Nielsen and G. K. McMaster, *J. Chromatogr.*, 402 (1987) 381.
25 M. W. Germann, R. T. Pon and J. H. V. D. Sande, *Anal. Biochem.*, 165 (1987) 399.
26 Y. Kato, T. Kitamura, A. Mitsui and T. Hashimoto, *J. Chromatogr.*, 398 (1987) 327.