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SEPARATION OF SEQUENCE ISOMERIC PYRIMIDINE OLIGODEOXYNUCLEOTIDES USING REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Nine mixtures of sequence isomeric pyrimidine oligonucleotides (dC, dT), (dC_2, dT), (dC, dT_2), (dC_3, dT), (dC_2, dT_2), (dC, dT_3), (dC_2, dT_3), (dC, dT_4) and (dC_3, dT_2), isolated from chemically depurinated and partially hydrolyzed herring sperm DNA, were separated totally or partially into their components using reversed-phase (Nucleosil C_{18}) high-performance liquid chromatography. The sequence and purity of the isolated pyrimidine oligonucleotides were determined by degradation using snake venom phosphodiesterase or by the "wandering spot" method. The results and advantages of the separation procedures are discussed with reference to the previous separation of the same mixtures using anion-exchange high-performance liquid chromatography.

INTRODUCTION

Pyrimidine oligonucleotides of identical chain length (isostisch[1]) can be isolated in preparative amounts from mixtures of partial hydrolysates of chemically depurinated DNA by the use of ion-exchange chromatography[2–5]. A further separation of mixtures of sequence isomeric pyrimidine oligonucleotides is achieved on the analytical scale by two-dimensional chromatography[6–10].

Different chromatographic steps combined with separation procedures are used for to process preparative amounts[11–20]. Moreover, it is possible to obtain almost complete separation of mixtures of sequence isomeric pyrimidine oligonucleotides up to pentamers into their components using anion-exchange high-performance liquid chromatography (HPLC) on analytical columns[21–23].

Up to now, the separation of preparative amounts has been difficult. On analytical columns the mixtures can be separated only in microgram amounts per passage, so it would be necessary, in order to work with larger amounts, either to use preparative columns or to repeat analytical separations several times under identical conditions. Usually the use of preparative columns is out of the question owing to their prohibitive cost. In addition to this it is uncertain how thoroughly the mixtures

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are fractionated on preparative columns. The consecutive separation of larger amounts using analytical ion-exchange columns is very intricate and time-consuming. After each run the column has to be equilibrated carefully, because gradients are used in the separation techniques. With respect to a preparative separation of pyrimidine oligonucleotides, we have investigated whether their sequence isomers can be separated by reversed-phase HPLC under isocratic conditions.

MATERIALS AND METHODS

Materials

Mixtures of sequence isomeric pyrimidine oligonucleotides were isolated from chemically degraded herring sperm DNA according to previously described methods[15,16,18]. Nucleosil C_{18}, particle size 7.5 μm (Macherey, Nagel & Co., Düren, F.R.G.) was used as stationary phase. Ammonium acetate, postassium dihydrogen phosphate and acetic acid, obtained from commercial sources, were of analytical grade and used without further purification. Methanol (LiChrosolv grade) was purchased from Merck (Darmstadt, F.R.G.). Buffer solutions were prepared using deionized and filtered water. The ammonium acetate buffer was adjusted to pH *ca.* 5 with acetic acid. Solvents A (buffer) and B (methanol–water, 60:40) were filtered and degassed by vacuum or sonication just before use.

Apparatus

A high-performance liquid chromatograph (Spectra-Physics SP 8000) was used together with a UV detector, operating at 254 nm. The analytical stainless-steel column (250 mm × 4.6 mm I.D.) was packed in our laboratory. All chromatographic procedures were performed at ambient temperature (19–22°C) and at a flow-rate of 1 ml/min. Nucleotides were detected by monitoring the effluent at 254 nm, and the peaks were integrated.

Characterization of isolated pyrimidine oligonucleotides

The isolated peak fractions were freeze-dried to remove ammonium acetate. An aliquot of the resulted lyophilysate was characterized by hydrolysis with snake venom phosphodiesterase followed by HPLC analysis of the nucleotides released as previously described[21–23]. The sequences of the isolated pyrimidine oligonucleotides listed in Table I were obtained using this procedure. The sequences of the other isolated pyrimidine oligonucleotides, however, were determined after labelling of the oligonucleotides using [γ-^{32}P]ATP and T_4 polynucleotide kinase by the well-known "wandering spot" sequence analysis[24–28]. The autoradiograms (fingerprints) of the pyrimidine oligonucleotides investigated by this procedure were also obtained.

RESULTS

DNA from herring sperm is chemically depurinated with formic acid in the presence of diphenylamine, followed by partial hydrolysis. The resulting mixture of pyrimidine nucleotides is fractionated according to the previously described separation procedure[15,16,18], whereby, amongst others, the following nine mixtures of sequence isomeric pyrimidine oligonucleotides are isolated chromatographically pure

TABLE I

RESULTS OF THE ENZYMATIC DEGRADATION OF PYRIMIDINE OLIGODEOXYNUCLEOTIDES USING SNAKE VENOM PHOSPHODIESTERASE

Pyrimidine nucleotides isolated from		*Degradation products*					*Concluded sequences*
Fig. No.	*Peak No.*	*Monomers (molar ratios)*				*Dimers*	
		dT	*dC*	*pdT*	*pdC*		
1	1	–	0.92	1.00	–	–	(dC-dT)
	2	1.00	–	–	1.00	–	(dT-dC)
2	1	–	0.91	2.00	–	(dC-dT)	(dC-dT-dT)
	2	1.00	–	0.94	0.96	(dT-dT)	(dT-dT-dC)
	3	1.00	–	1.03	0.97	(dT-dC)	(dT-dC-dT)
3	1	–	1.00	0.98	1.04	(dC-dT)	(dC-dT-dC)
	2	–	1.00	0.94	0.98	(dC-dC)	(dC-dC-dT)
	3	1.00	–	–	1.96	(dT-dC)	(dT-dC-dC)
4	1	–	1.00	2.89	–	(dC-dT)	(dC-dT-dT-dT)
5	2	–	1.00	0.94	2.08	(dC-dT)	(dC-dT-dC-dC)

and in preparative amounts: (dC, dT); (dC, dT_2); (dC_2, dT); (dC, dT_3);(dC_3, dT); (dC_2, dT_2); (dC, dT_4); (dC_2, dT_3); (dC_3, dT_2). The mixtures are then partially or totally separated into their components on an analytical reversed-phase column (Nucleosil C_{18}) by HPLC. The solvent system used for elution consists of two components. Component A is a 0.1 *M* ammonium acetate solution (pH *ca.* 5) except for the separation of (dC, dT_2) which is performed using 0.01 *M* potassium dihydrogen phosphate (pH *ca.* 5). Component B consists of methanol–water (60:40, v/v). The mixtures of isomers of pyrimidine oligonucleotides are separated under isocratic conditions, except the isomers (dC, dT_4) and (dC_3, dT_2). The eluent composition varies for the different oligonucleotides. Separation of isomers of the two mixtures of the pyrimidine oligonucleotides mentioned above requires a gradient with a linear increase of component B. The elution profiles measured by adsorption at 254 nm are shown in Figs. 1–9. Retention times and peak area percentages are summarized in Table II.

In order to isolate the different isomers eluted, the fractions of the respective peaks are pooled and lyophilysed. The purity and sequence of the low-molecular-weight pyrimidine oligonucleotides are determined from their degradation products (monomers, dimers) according to the procedure described extensively elsewhere[21–23]. This degradation is performed by a partial or total hydrolysis using snake venom phosphodiesterase. The results are summarized in Table I.

The higher-molecular-weight pyrimidine oligonucleotides are characterized by the well-known "wandering spot" method. For this, aliquots of isolated pyrimidine oligonucleotides are first enzymatically labelled in the 5'-hydroxyl group using [γ-^{32}P]ATP and T_4 polynucleotide kinase. The labelled oligonucleotides are then also partially hydrolysed with snake venom phosphodiesterase. The resulting hydrolysate is chromatographed two-dimensionally. From the autoradiograms (fingerprints), which are obtained after chromatography, the purity as well as the sequence of the pyrimidine oligonucleotides can be determined (Fig. 10).

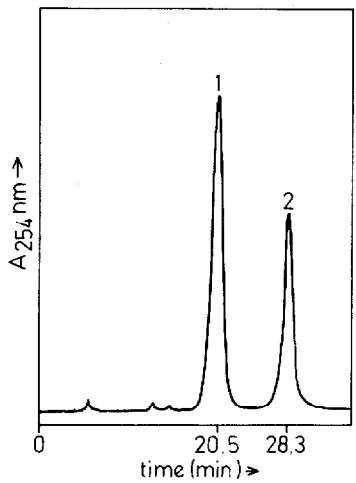
TABLE II

CONDITIONS AND RESULTS OF THE REVERSED-PHASE HPLC OF MIXTURES OF SEQUENCE ISOMERIC PYRIMIDINE OLIGODEOXYNUCLEOTIDES

Chromatographed mixtures of sequence isomers (A_{260}-units; µl)	*Eluent**		*Separated pyrimidine oligodeoxynucleotides*					*Fingerprint (see Fig. 10)*
	A(%)	*B(%)*	*Fig. No.*	*Peak No.*	*Retention time (min)*	*Peak area (%)*	*Sequence of the oligonucleotides*	
(dC, dT)	86	14	1	1	20.5	60.17	(dC-dT)***	–
34.5; 100	isocratic		1	2	28.3	37.66	(dT-dC)***	–
(dC, dT_2)	73**	27	2	1	10.0	21.65	(dC-dT-dT)***	–
4.5; 30	isocratic		2	2	15.3	29.94	(dT-dT-dC)***	–
			2	3	20.2	46.46	(dT-dC-dT)***	–
(dC_2, dT)	80	20	3	1	28.1	29.39	(dC-dT-dC)***	–
7.8; 60	isocratic		3	2	35.1	42.02	(dC-dC-dT)***	–
			3	3	43.4	27.55	(dT-dC-dC)***	–
(dC, dT_3)	78	22	4	1	11.9	33.31	(dC-dT-dT-dT)***	–
0.5; 3	isocratic		4	2	18.3	11.47	(dT-dT-dT-dC)	a
			4	3	19.1	15.01	(dT-dT-dC-dT)	b
			4	4	20.3	35.28	(dT-dC-dT-dT)	c
(dC_3, dT)	66	34	5	1	11.1	44.06	(dC-dC-dT-dC) } (dC-dC-dC-dT) }	d
0.9; 3	isocratic							
			5	2	12.9	44.64	(dC-dT-dC-dC)***	–
			5	3	14.5	10.02	(dT-dC-dC-dC)	e
			6	1	12.8	26.40	(dC-dT-dC-dT)	f
(dC_2, dT_2)	74	26	6	2	13.7	14.72	(dC-dC-dT-dT)	g
3.0; 10	isocratic		6	3	14.5	17.27	(dC-dT-dT-dC)	h
			6	4	16.3	12.78	(dT-dC-dT-dC)	i
			6	5	18.7	20.14	(dT-dC-dC-dT) } (dT-dT-dC-dC) }	j

(dC_4, dC_4)	B increased linearly	7	1	10.9	17.00	(dC-dT-dT-dT-dT)	k
0.5; 3	during 30 min	7	2	15.5	11.58	(dT-dC-dT-dT-dT)	l
	from 23 to 27	7	3	16.2	13.59	(dT-dT-dC-dT-dT)	m
		7	4	17.7	14.85	(dT-dT-dT-dC-dT)	n
		7	5	18.3	17.38	(dT-dT-dT-dT-dC)	
(dC_2, dT_3)	73 27	8	1	6.1	2.85	Impurities	–
0.9; 3	isocratic	8	2	15.0	20.16	(dC-dT-dT-dC-dT)	o
						(dC-dT-dC-dT-dT)	
						(dC-dC-dT-dT-dT)	
						(dT-dC-dC-dT-dT)	
		8	3	17.1	6.72	(dC-dT-dT-dT-dC)	p
		8	4	20.3	18.46	(dT-dC-dT-dC-dT)	q
		8	5	21.6	17.20	(dT-dT-dT-dC-dC)	r
						(dT-dT-dC-dT-dC)	
		8	6	24.1	20.74	(dT-dT-dC-dC-dT)	s
		8	7	25.8	11.08	(dT-dC-dT-dT-dC)	t
(dC_3, dT_2)	B increased	9	1	8.4	1.78	Impurities	–
1.5; 10	linearly during	9	2	9.6	11.44	Impurities	–
	30 min from	9	3	11.8	33.30	(dC-dT-dC-dT-dC)	u
	27 to 32					(dC-dT-dC-dC-dT)	
		9	4	12.9	8.85	(dC-dC-dT-dT-dC)	v
						(dC-dC-dT-dC-dT)	
						(dC-dT-dT-dC-dC)	
						(dC-dT-dC-dC-dT)	
		9	5	15.1	11.92	(dT-dC-dC-dT-dC)	w
						(dT-dC-dT-dC-dC)	
		9	6	16.3	18.39	(dT-dC-dT-dC-dC)	x
						(dT-dC-dC-dT-dC)	
		9	7	21.8	8.33	Mixture, not determined	–
		9	8	23.4	3.99		–

* A = 0.1 *M* ammonium acetate (pH 5); B = methanol–water, 60:40.
** A = 0.01 *M* potassium dihydrogen phosphate (pH 5).
*** Concluded from the results of enzymatic degradation using snake venom phosphodiesterase (see Table I).



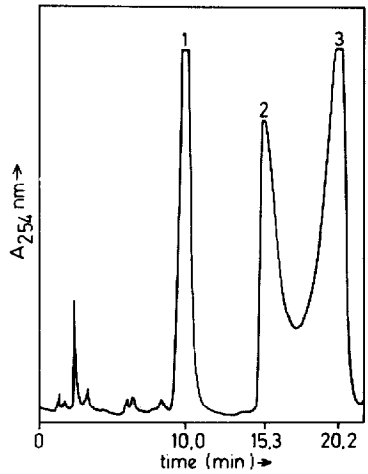
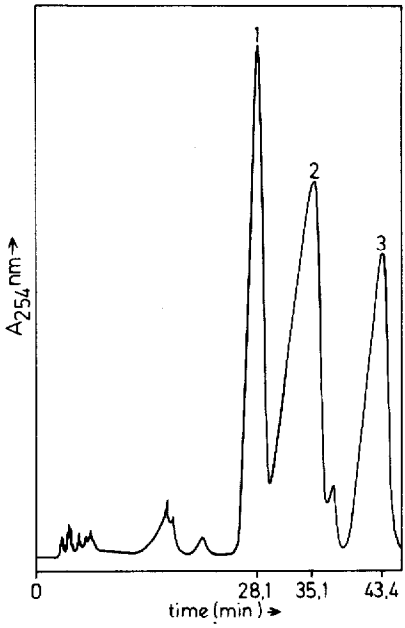


Fig. 1. Separation of (dC, dT) (34.5 A_{260}-units) on Nucleosil C_{18} (7.5 μm) column (250 × 4.6 mm I.D.) by isocratic elution with 86% A (A = 0.1 *M* ammonium acetate) and 14% B (B = methanol–water, 60:40) at room temperature; flow-rate, 1 ml/min. Peaks: 1 = (dC-dT); 2 = (dT-dC).

Fig. 2. Separation of (dC, dT_2) (4.5 A_{260}-units) on a Nucleosil C_{18} (7.5 μm) column (250 × 4.6 mm I.D.) by isocratic elution with 73% A (A = 0.01 *M* potassium dihydrogen phosphate) and 27% B (B = methanol–water, 60:40) at room temperature; flow-rate, 1 ml/min. Peaks: 1 = (dC-dT-dT); 2 = (dT-dT-dC); 3 = (dT-dC-dT).



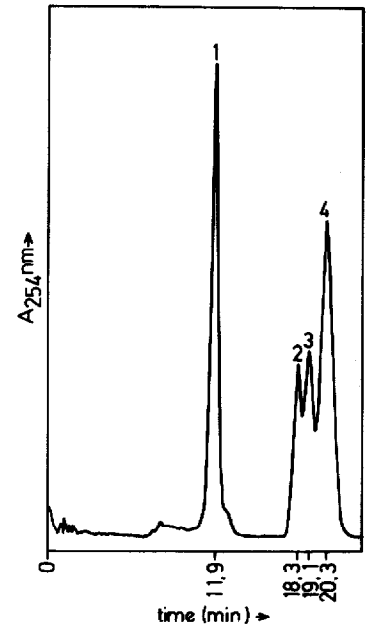


Fig. 3. Separation of (dC_2, dT) (7.8 A_{260}-units) on a Nucleosil C_{18} (7.5 μm) column (250 × 4.6 mm I.D.) by isocratic elution with 80% A (A = 0.1 *M* ammonium acetate) and 20% B (B = methanol–water, 60:40) at room temperature; flow-rate, 1 ml/min. Peaks: 1 = (dC-dT-dC); 2 = (dC-dC-dT); 3 = (dT-dC-dC).

Fig. 4. Separation of (dC, dT_3) (0.5 A_{260}-units) on a Nucleosil C_{18} (7.5 μm) column (250 × 4.6 mm I.D.) by isocratic elution with 78% A (A = 0.1 *M* ammonium acetate) and 22% B (B = methanol–water, 60:40) at room temperature; flow-rate 1 ml/min. Peaks: 1 = (dC-dT-dT-dT); 2 = (dT-dT-dT-dC); 3 = (dT-dT-dC-dT); 4 = (dT-dC-dT-dT).

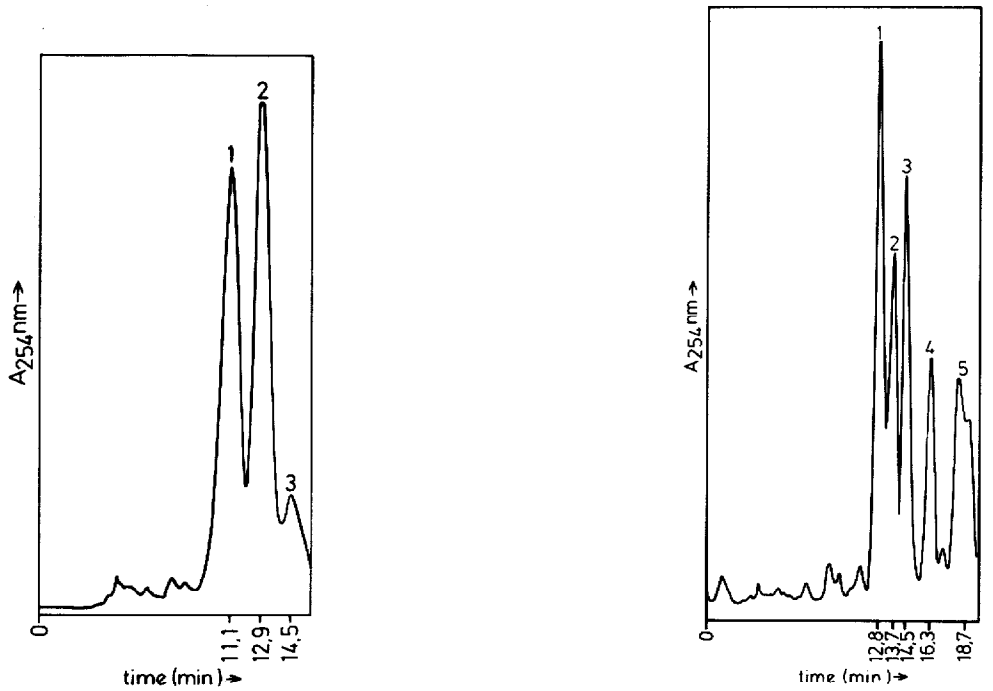


Fig. 5. Separation of (dC_3, dT) (0.9 A_{260}-units) on a Nucleosil C_{18} (7.5 μm) column (250 × 4.6 mm I.D.) by isocratic elution with 66% A (A = 0.1 *M* ammonium acetate) and 34% B (B = methanol–water, 60:40) at room temperature; flow-rate, 1 ml/min. Peaks: 1 = (dC-dC-dC-dT) and (dC-dC-dT-dC); 2 = (dC-dT-dC-dC); 3 = (dT-dC-dC-dC).

Fig. 6. Separation of (dC_2, dT_2) (3.0 A_{260}-units) on a Nucleosil C_{18} (7.5 μm) column (250 × 4.6 mm I.D.) by isocratic elution with 74% A (A = 0.1 *M* ammonium acetate) and 26% B (B = methanol–water, 60:40) at room temperature; flow-rate, 1 ml/min. Peaks: 1 = (dC-dT-dC-dT); 2 = (dC-dC-dT-dT); 3 = (dC-dT-dT-dC); 4 = (dT-dC-dT-dC); 5 = (dT-dC-dC-dT) and (dT-dT-dC-dC).

HPLC of the nine mixtures of sequence isomeric pyrimidine oligonucleoides leads to the following results. The mixture of dimers (dC, dT) is completely separated into its two components: (dC-dT) is eluted prior to (dT-dC) (Fig. 1). The proportion of (dC-dT) in the mixture is significantly higher than that of (dT-dC) (60:38).

The mixture of trimers (dC, dT_2) and (dC_2, dT) is also well separated into their respective three components (Figs. 2 and 3). The trinucleoside diphosphate (dT-dC-dT), at 46%, constitutes the main component of (dC, dT_2), whereas the other two sequence isomers (dC-dT-dT) and (dT-dT-dC) amount to 22% and 30%, respectively. The mixture (dC_2, dT) consists of 42% of (dC-dC-dT) and 28% of each of the trinucleoside diphosphates (dC-dT-dC) and (dT-dC-dC). The sequence of the isolated trinucleoside diphosphates follows clearly from the molar ratios of the monomer units, which are determined by enzymatic total hydrolysis, and from the sequence of the dimers, which appear after enzymatic partial hydrolysis (Table I).

From the mixture (dC, dT_3) peak 1 (dC-dT-dT-dT) is clearly separated from the remaining sequence isomers, which are only partially separated (Fig. 4). The sequence (dC-dT-dT-dT), which constitutes 33% of the mixture, is derived from the

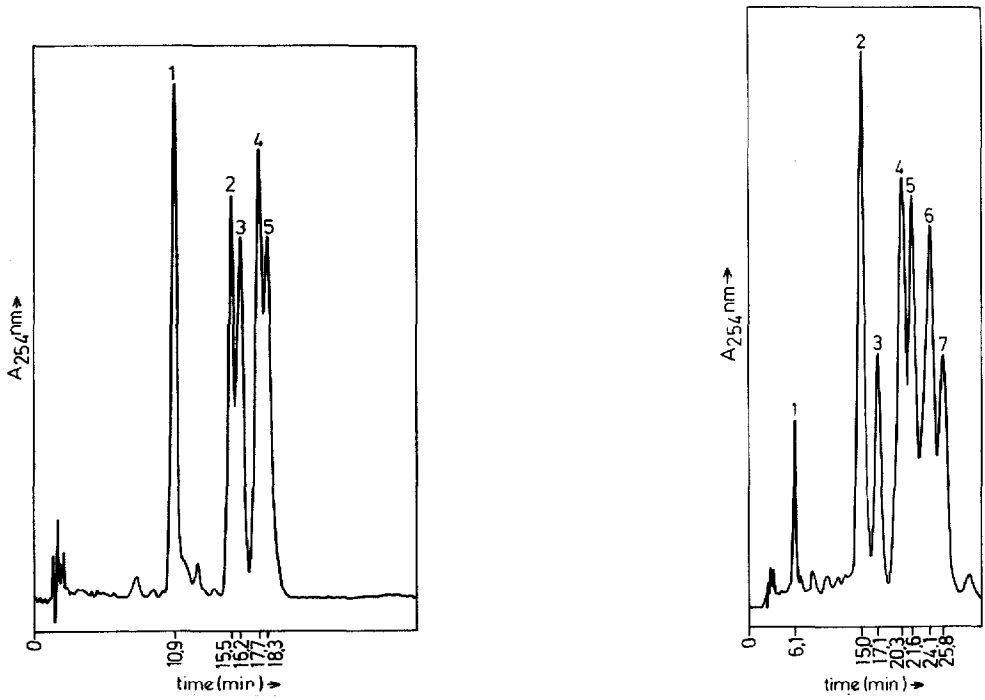


Fig. 7. Separation of (dC, dT_4) (0.5 A_{260}-units) on Nucleosil C_{18} (7.5 μm; 250 × 4.6 mm I.D.) with a linear gradient from 23% to 27% B within 30 min at room temperature (A = 0.1 *M* ammonium acetate; B = methanol–water, 60:40; flow-rate, 1 ml/min). Peaks: 1 = (dC-dT-dT-dT-dT); 2 = (dT-dC-dT-dT-dT); 3 = (dT-dT-dC-dT-dT); 4 and 5 = (dT-dT-dT-dC-dT) and (dT-dT-dT-dT-dC).

Fig. 8. Separation of (dC_2, dT_3) (0.8 A_{260}-units) on a Nucleosil C_{18} (7.5 μm) column (250 × 4.6 mm I.D.) by isocratic elution with 73% A (A = 0.1 *M* ammonium acetate) and 27% B (B = methanol water, 60:40) at room temperature; flow-rate, 1 ml/min. Peaks: 1 = impurities; 2 = (dC-dT-dT-dC-dT), (dC-dT-dC-dT-dT), (dC-dC-dT-dT-dT) and (dT-dC-dC-dT-dT); 3 = (dC-dT-dT-dT-dC); 4 = (dT-dC-dT-dC-dT); 5 = (dT-dT-dT-dC-dC) and (dT-dT-dC-dT-dC); 6 = (dT-dT-dC-dC-dT); 7 = (dT-dC-dT-dT-dC).

enzymatic degradation data (Table I). The sequences of the three other isomers are determined from their fingerprints (Fig. 10a–c). The fingerprints show that these sequence isomers are isolated chromatographically pure, although they are not eluted in completely distinct peaks. Peak 2 contains (dT-dT-dT-dC), peak 3 (dT-dT-dC-dT) and peak 4 (dT-dC-dT-dT). The percentages of these in (dC, dT_3) amount to 11%, 15% and 35%, respectively.

(dC_3, dT) is separated into only three peaks under isocratic conditions (Fig. 5). The fingerprint (Fig. 10d) of peak 1, which contains 44% of the mixture, shows that (dC-dC-dT-dC) is eluted together with (dC-dC-dC-dT). Peak 2, which also amounts to 44% of the mixture, contains pure (dC-dT-dC-dC), as the enzymatic hydrolysis with phosphodiesterase proves (Table I). In peak 3, according to the fingerprint (Fig. 10e), (dT-dC-dC-dC) is eluted, as 10% of (dC_3, dT).

The sequence isomeric tetranucleoside triphosphates (dC_2, dT_2) are eluted under isocratic conditions in six more-or-less well separated main peaks (Fig. 6).

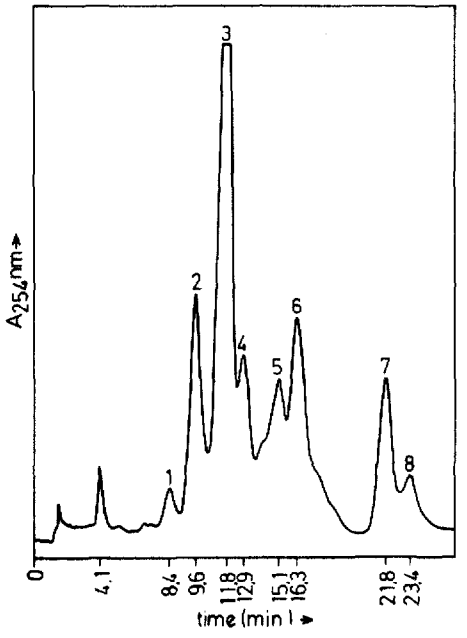
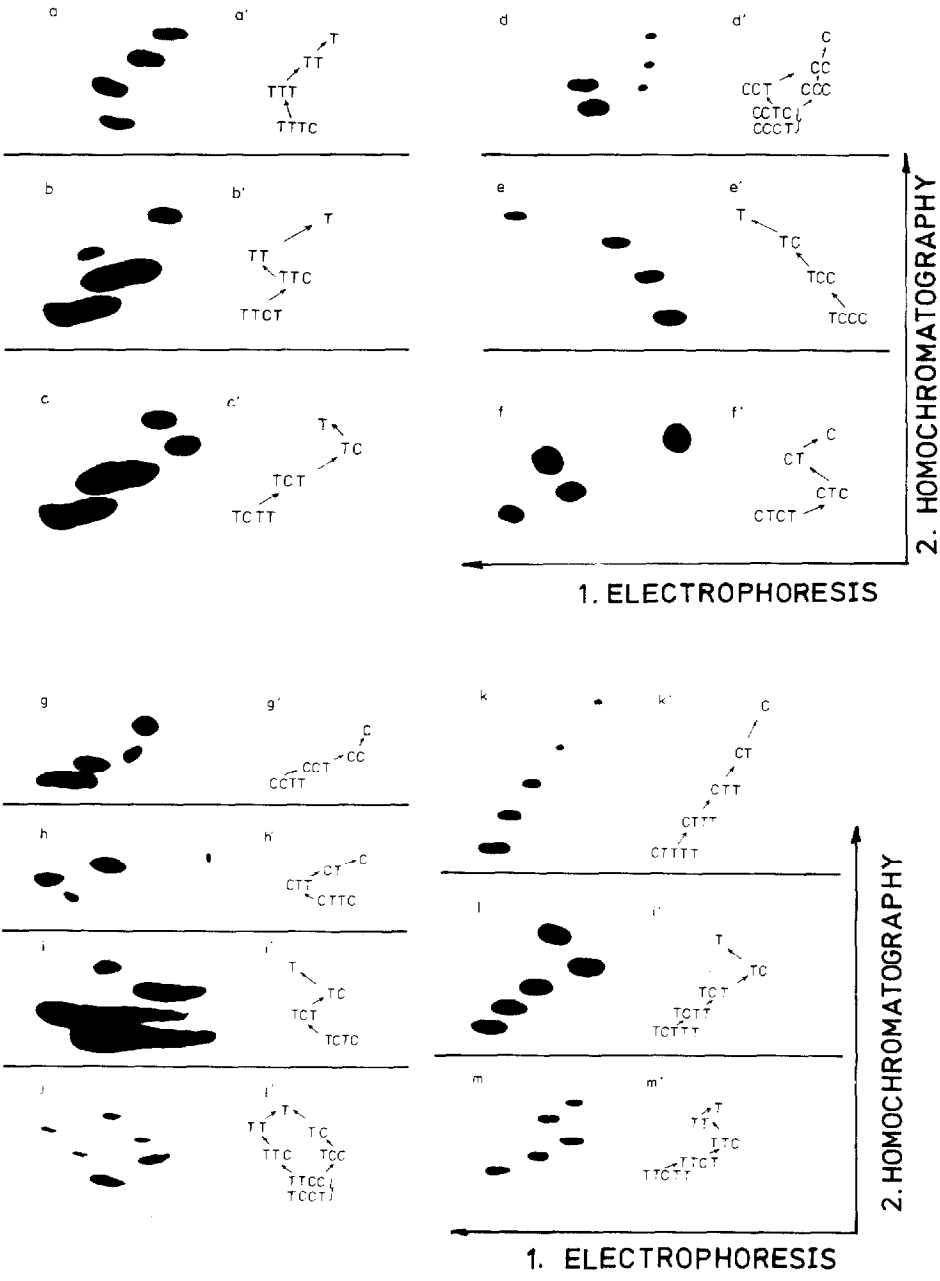


Fig. 9. Separation of (dC_3, dT_2) (1.5 A_{260}-units) on Nucleosil C_{18} (7.5 μm; 250 × 4.6 mm I.D.) with a linear gradient from 27% to 32% B within 30 min at room temperature (A = 0.1 *M* ammonium acetate; B = methanol–water, 60:40); flow-rate, 1 ml/min. Peaks: 1 and 2 = impurities; 3 = (dC-dT-dC-dT-dC) and (dC-dT-dC-dC-dT); 4 = (dC-dC-dT-dT-dC), (dC-dC-dT-dC-dT), (dC-dT-dT-dC-dC) and (dC-dT-dC-dC-dT); 5 = (dT-dC-dC-dT-dC) and (dT-dC-dT-dC-dC); 6 = (dT-dC-dT-dC-dC) and (dT-dC-dC-dT-dC); 7 and 8 = unidentified mixtures.

Peak 1 contains 26% of the mixture and is (dC-dT-dC-dT) according to the fingerprint (Fig. 10f). Peaks 2, 3 and 4, (dC-dC-dT-dT), (dC-dT-dT-dC) and (dT-dC-dT-dC) are each isolated in chromatographically pure form (Fig. 10g–i). Because the peaks of these isomers are not completely separated from each other, the overlapping fractions are discarded. The three sequence isomers amount to 14%, 17% and 13% of (dC_2, dT_2), respectively. In the double peak 5, which contains 20% of the mixture, (dT-dC-dC-dT) is eluted with (dT-dT-dC-dC). It can be seen from the fingerprint (Fig. 10j) that there is more (dT-dC-dC-dT) than (dT-dT-dC-dC).

(dC, T_4) is separated into five main peaks using a linear gradient. All peaks together contain 75% of the amount of the mixture applied (Fig. 7). Peak 1, 17%, is clearly separated from the remaining four peaks and contains (dC-dT-dT-dT-dT) in chromatographically pure form (Fig. 10k). From peaks 2 and 3, also in pure form, the sequence isomers (dT-dC-dT-dT-dT) and (dT-dT-dC-dT-dT), respectively, are isolated (Fig. 10l and m), and amount to 12% and 14%. Some 32% of the mixture consists of the remaining two sequence isomers from (dC, dT_4), namely the pentanucleoside tetraphosphates (dT-dT-dT-dT-dC) and (dT-dT-dT-dC-dT), eluted in peaks 4 and 5. The fingerprint (Fig. 10n) shows that they are not isolated chromatographically pure.

The isocratic separation of (dC_2, dT_3), which theoretically contains ten different sequence isomers, results in seven main peaks (Fig. 8), which together contain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TTTC
d
d'
C
CC
CCT
CCC
CCTC
CCCT
b
b'
T
TT
TTC
TTCT
e
e'
T
TC
TCC
TCCC
c
c'
T
TC
TCT
TCTT
f
f'
C
CT
CTC
CTCT
2. HOMOCHROMATOGRAPHY
1. ELECTROPHORESIS
g
g'
C
CC
CCT
CCTT
h
h'
C
CT
CTT
CTTC
i
i'
T
TC
TCT
TCTC
j
j'
T
TT
TC
TTC
TCC
TTCC
TCCT
k
k'
C
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CTT
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CTTTT
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TCTTT
m
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2. HOMOCHROMATOGRAPHY
1. ELECTROPHORESIS

Fig. 10.

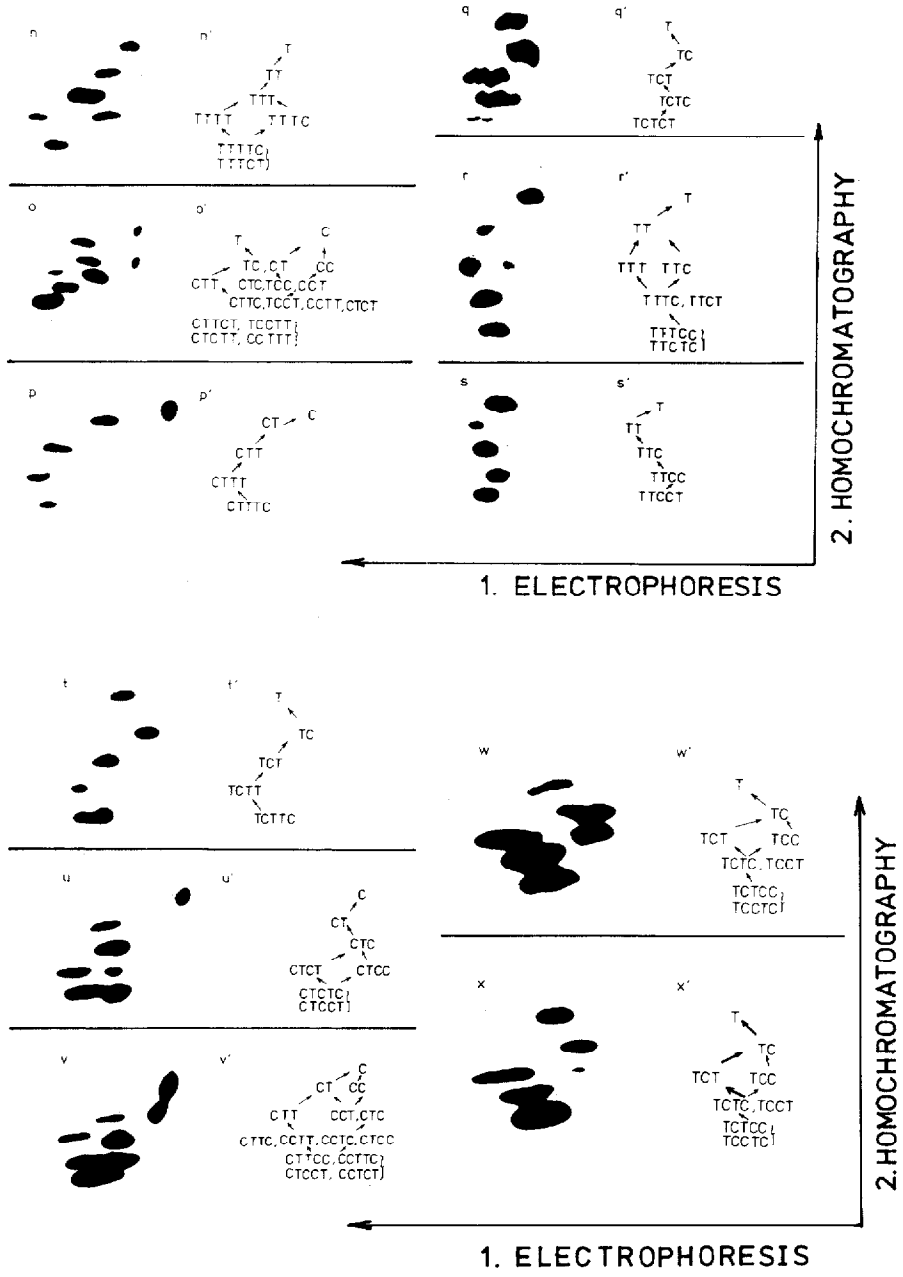

Fig. 10. Determination of the sequence of pyrimidine oligodeoxynucleotides isolated from mixtures of sequence isomers of pyrimidine oligonucleotides from herring sperm DNA using reversed-phase HPLC. The isolated oligonucleoside phosphates are first labelled with ^{32}P and then partially hydrolysed with snake venom phosphodiesterase. The resulting partial hydrolysate is two-dimensionally chromatographed. The first dimension consists of an electrophoretic separation on cellulose acetate strips at pH 3.5. A homochromatography on DEAE-cellulose thin-layer plates represents the second dimension. (a–x) show the resulting autoradiograms, and (a′–x′) show the interpretations of the fingerprints.

more than 97%. It is true that all ten sequence isomeric pentanucleoside tetraphosphates of (dC_2, dT_3) can be identified in the eluate on the evidence of the fingerprints (Fig. 10o–t), but only four can be isolated in chromatographically pure form. Investigation of the lyophilysates of the seven peaks leads to the following results. Peak 1 contains impurities which were not further identified. In peak 2, *ca.* 20% of the mixture according to the fingerprint (Fig. 10o), the four sequence isomers (dC-dT-dT-dC-dT), (dC-dT-dC-dT-dT), (dC-dC-dT-dT-dT) and (dT-dC-dC-dT-dT) are eluted. Peak 3 contains chromatographically pure (dC-dT-dT-dT-dC) (Fig. 10p), amounting to 7% of the mixture. The sequence isomer (dT-dC-dT-dC-dT) (18% of the mixture) is isolated pure from peak 4 (Fig. 10q). In peak 5, which contains 17%, the two sequence isomers (dT-dT-dT-dC-dC) and (dT-dT-dC-dT-dC) are co-eluted, as can be estimated from the fingerprint (Fig. 10r). Peak 6 contains 21% pure (dT-dT-dC-dC-dT) (Fig. 10s). Peak 7 contains 11% of the mixture which, according to the fingerprint (Fig. 10t), consists of the (dT-dC-dT-dT-dC) in pure form.

Fractionation of (dC_3, dT_2) using a linear gradient produces eight relatively broad main peaks (Fig. 9), which together contain more than 97% of the amount applied. From the ten theoretically possible sequence isomers, seven can be identified in the different peaks on the evidence of the fingerprints (Fig. 10u–x). The sequence isomers (dC-dC-dC-dT-dT), (dT-dT-dC-dC-dC) and (dT-dC-dC-dC-dT) are not detected. The separation of this mixture is not satisfactory, because no sequence isomers can be isolated as a single substance. The investigation of lyophilysates of the eight peaks leads to the following results. Peaks 1 and 2 contain unidentified impurities. In peak 3, which comes to 33% of the mixture, the sequence isomers (dC-dT-dC-dT-dC) and (dC-dT-dC-dC-dT) are eluted together (Fig. 10u). (dC-dT-dC-dC-dT) is also found in peak 4 besides the three other sequence isomers (dC-dC-dT-dT-dC), (dC-dC-dT-dC-dT) and (dC-dT-dT-dC-dC), as the fingerprint (Fig. 10v) shows. This is because peak 4, which comprises 9%, is only partially separated from peak 3. Peaks 5 and 6, which contain 12% and 18%, respectively, of the mixture, are also eluted so close together that no isolation of single sequence isomers is possible. As the fingerprints (Fig. 10w and x) show, the sequence isomers (dT-dC-dC-dT-dC) and (dT-dC-dT-dC-dC) each are isolated in slightly different amounts from both peaks. The lyophilysates of peaks 7 and 8, which together contain 12% of the mixture, do not yield fingerprints which can be clearly interpreted. But the possibility cannot be excluded that the undetected sequence isomers are eluted in these peaks.

DISCUSSION

The results show that mixtures of sequence isomeric pyrimidine oligonucleotides up to pentamers are almost completely separated into their components by reversed-phase HPLC. From the 47 various sequence isomers which theoretically can be contained in the nine investigated mixtures, 44 were identified. From these, 25 pyrimidine oligonucleotides were isolated as pure substances, the remaining nineteen as mixtures in preparative amounts. The fingerprints show clearly that even components that are not separated into distinct peaks, still can be isolated chromatographically pure. The best separation results are obtained by HPLC on Nucleosil C_{18} columns. Considerably worse results are produced with other comparable materials which are offered by other producers. In general, for the quality of separation

TABLE III

PYRIMIDINE OLIGODEOXYNUCLEOTIDES ISOLATED FROM MIXTURES OF SEQUENCE ISOMERS USING HPLC

*Mixture of sequence isomeric pyrimidine oligodeoxynucleotides**	*Compounds theoretically contained in the mixture*	*Compounds isolated (reversed-phase HPLC)*		*Compounds isolated (anion-exchange HPLC)*		*Ref. No.*
		As a single substance	*As a mixture*	*As a single substance*	*As a mixture*	
(dC, dT)	1. (dC-dT)	+	–	–	+	21
	2. (dT-dC)	+	–	–	+	21
(dC, dT_2)	1. (dC-dT-dT)	+	–	+	–	21
	2. (dT-dC-dT)	+	–	+	–	21
	3. (dT-dT-dC)	+	–	+	–	21
(dC_2, dT)	1. (dC-dC-dT)	+	–	+	–	21
	2. (dC-dT-dC)	+	–	–	+	21
	3. (dT-dC-dC)	+	–	–	+	21
(dC, dT_3)	1. (dC-dT-dT-dT)	+	–	+	–	22
	2. (dT-dT-dT-dC)	+	–	+	–	22
	3. (dT-dC-dT-dT)	+	–	+	–	22
	4. (dT-dT-dC-dT)	+	–	+	–	22
(dC_3, dT)	1. (dC-dC-dC-dT)	–	+	+	–	22
	2. (dC-dC-dT-dC)	–	+	+	–	22
	3. (dC-dT-dC-dC)	+	–	+	–	22
	4. (dT-dC-dC-dC)	+	–	+	–	22
(dC_2, dT_2)	1. (dC-dC-dT-dT)	+	–	+	–	22
	2. (dC-dT-dC-dT)	+	–	+	–	22
	3. (dT-dC-dC-dT)	–	+	+	–	22
	4. (dT-dT-dC-dC)	–	+	+	–	22
	5. (dC-dT-dT-dC)	+	–	+	–	22
	6. (dT-dC-dT-dC)	+	–	+	–	22
(dC, dT_4)	1. (dC-dT-dT-dT-dT)	+	–	+	–	23
	2. (dT-dC-dT-dT-dT)	+	–	+	–	23
	3. (dT-dT-dC-dT-dT)	+	–	+	–	23
	4. (dT-dT-dT-dC-dT)	–	+	+	–	23
	5. (dT-dT-dT-dT-dC)	–	+	+	–	23
(dC_2, dT_3)	1. (dC-dC-dT-dT-dT)	–	+	+	–	23
	2. (dC-dT-dT-dT-dC)	+	–	–	+	23
	3. (dT-dT-dT-dC-dC)	–	+	+	–	23
	4. (dC-dT-dC-dT-dT)	–	+	+	–	23
	5. (dT-dC-dT-dT-dC)	+	–	–	+	23
	6. (dT-dC-dT-dC-dT)	+	–	–	+	23
	7. (dT-dT-dC-dC-dT)	+	–	–	+	23
	8. (dT-dC-dC-dT-dT)	–	+	+	–	23
	9. (dT-dT-dC-dT-dC)	–	+	+	–	23
	10. (dC-dT-dT-dC-dT)	–	+	+	–	23
(dC_3, dT_2)	1. (dC-dC-dC-dT-dT)	–	–	+	–	23
	2. (dC-dT-dC-dC-dT)	–	+	–	–	23
	3. (dC-dT-dT-dC-dC)	–	+	–	–	23
	4. (dT-dT-dC-dC-dC)	–	–	+	–	23
	5. (dT-dC-dC-dC-dT)	–	–	–	–	23
	6. (dT-dC-dT-dC-dC)	–	+	+	–	23
	7. (dT-dC-dC-dT-dC)	–	+	–	–	23
	8. (dC-dT-dC-dT-dC)	–	+	–	–	23
	9. (dC-dC-dT-dT-dC)	–	+	–	–	23
	10. (dC-dC-dT-dC-dT)	–	+	+	–	23

* Isolated from chemically degraded herring sperm DNA.

it is not important whether 0.1 *M* ammonium acetate or 0.01 *M* potassium dihydrogen phosphate is used as eluent A. However, a much better separation of (dC, dT_2) was observed when 0.01 *M* potassium dihydrogen phosphate was used. For preparative separation ammonium acetate is to be preferred, because it can be removed form the collected fractions more easily.

In comparing our results with previous data obtained from on ion-exchange columns (Table III), significant differences can be observed. With both techniques only the mixtures (dC, dT_2) and (dC, dT_3) were completely separated although the elution orders of the components differ.(dC, dT) and (dC_2, dT), which are well separated on Nucleosil C_{18}, undergo only partial separation on the ion-exchange column. The reverse is observed for the separation of (dC_3, dT_3), (dC_2, dT_2) and (dC, dT_4): the ion-exchange column effects a complete separation whereas on Nucleosil C_{18} only a partial separation is possible. Only the combination of both techniques results in a complete separation of the isomers of (dC_2, dT_3). Sequence isomers which are co-eluted on ion-exchange columns are eluted on Nucleosil C_{18} as pure components and *vice versa*. Only a partial separation of (dC_3, dT_2), is possible even if both techniques are combined. Whereas on ion-exchange materials four sequence isomers can be isolated, no pure isomer can be observed after separation on Nucleosil C_{18}. It is remarkable that the pentanucleoside tetraphosphate (dT-dC-dC-dC-dT) is found in none of the separations. Possibly it is present either in only very small concentrations or not at all in the mixture investigated.

This relatively simple method for the separation of sequence isomeric oligonucleotides can be applied to isolate numerous oligodeoxynucleotides of defined sequence that previously were obtainable only via very complicated chemical synthesis.

Although the separation results on Nucleosil C_{18} are similar to those on ion-exchange materials, reversed-phase HPLC offers the following advantages in dealing with larger quantities. Seven out of nine mixtures are separated satisfactorily on Nucleosil C_{18} under isocratic conditions. Larger amounts of these mixtures can therefore be processed step by step in smaller portions on analytical columns by repeated separation. A corresponding processing using a gradient, which is used with anion-exchange HPLC, would be far more complicated. Moreover, separations on Nucleosil C_{18} columns are carried out under milder conditions (pH *ca.* 5; room temperature), whereas the anion-exchange columns are operated under more drastic conditions (pH 3.5; 70°C) when part of the pyrimidine oligonucleotides may be destroyed.

The present method, apart from the new possibility of obtaining certain pyrimidine oligonucleotides chromatographically, also offers the following aspect. The separation of natural mixtures of sequence isomeric oligodeoxynucleotides, which are very easily accessible, also yields information on the frequency of certain sequences in genetic material. Therefore, HPLC presents a new simple way to compare DNA of various origins, relative to certain sequences. This is only possible at great expense using previous methods.

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