CHROM. 21 411

QUANTITATIVE EXTRACTION OF THYMINE–THYMINE DIMER FROM A LARGE EXCESS OF THYMINE BY PREPARATIVE LIQUID CHROMATOGRAPHY

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SUMMARY

Extremely small amounts of thymine–thymine dimer can be extracted from thymine solutions using overloaded elution and shaving a narrow fraction at the front of the overloaded, later eluting thymine band. A two- or three-step procedure, involving the concentration and reinjection of the collected fraction, permits the preparation of solutions in which the ratio of the concentrations of the dimer and the monomer is close to unity compared with less than 1:25 000 in the original samples.

The theory of non-linear chromatography and the semi-ideal model permits the accurate prediction of the location of the trace component band and the determination of the appropriate time to start and stop collection of the enriched fraction.

INTRODUCTION

Exposure of deoxyribonucleic acids in living cells to ultraviolet radiation results in damaging structural alterations. The reported chemical lesions in the DNA material of some animal and plant systems include classes of pyrimidine cyclobutane dimers, pyrimidine hydrates, glycols, various addition products and some purine photoproducts[1]. The pyrimidine cyclobutane dimers (thymine–thymine, thymine–cytosine and cytosine–cytosine) are the most abundant, representing as much as 90% of the UV damage[1]. With physiologically significant exposures (*e.g.*, less than 10 J/m^2 at 254 nm), the concentration of these compounds is extremely low, of the order of 0.02% or less of the base concentration[2].

The accurate determination of these trace constituents requires adequate separation from the large amounts of the normal bases. A complete analytical procedure

involves the quantitative extraction of the dimers from a mixture of regular bases, followed by derivatization of the dimers and their quantitative analysis by gas chromatography with electron-capture detection. This scheme makes possible the achievement of very low detection limits in spite of the small size of the samples available, which do not exceed 500 μg.

To aid in the selection of optimum conditions for the isolation and determination of trace amounts of thymine–thymine dimer, changes in the elution profile of this dimer on a high-performance liquid chromatographic (HPLC) column overloaded with thymine have been examined both experimentally and theoretically, using the semi-ideal model of non-linear chromatography described previously[3,4].

EXPERIMENTAL

Analyses were performed on a Waters liquid chromatographic system equipped with a Model 600E multi-solvent delivery system, a Model U6K injector and a Model 490 programmable multi-wavelength detector (Waters Chromatography Division, Millipore, Milford, MA, U.S.A.). Data were acquired at 210 and 300 nm by a Maxima 820 chromatography workstation (Dynamic Solutions, Millipore, Ventura, CA, U.S.A.).

The linear range of the UV detector used is not sufficient to permit detection of the monomer and the dimer simultaneously at the same wavelength, owing to the very low concentration of the latter in the samples. Accordingly, thymine–thymine dimer is monitored at 210 nm, because its response remains within the linear range of the detector, whereas thymine monomer is detected at 300 nm, where the absorbance is small enough to provide a linear response in the concentration range investigated. Two calibration graphs were determined.

An Alltech C_{18} HS (7 μm) column (250 mm × 4.6 mm I.D.) was used for the separations. High-purity water (Burdick & Jackson Labs., Muskegon, MI, U.S.A.), filtered through a 0.45-μm Nylon 66 membrane (Supelco, Bellefonte, PA, U.S.A.), was used for the mobile phase. Data were obtained at ambient temperature at a flow-rate of 1.0 ml/min.

Thymine was purchased from Sigma (St. Louis, MO, U.S.A.) and the *cis-syn*-5,6-thymine–thymine dimer was prepared by modifying the procedure of Wang[5], irradiating (at 254 nm) a frozen 0.1% aqueous solution of thymine. A white crystalline product was obtained and determined to be 99% pure dimer by HPLC. All standards were prepared in aqueous solution and calibration graphs were constructed from the peak heights at different concentrations.

The adsorption equilibrium isotherm of thymine in the chromatographic system used was measured by the method of elution by characteristic point (ECP)[6,7].

THEORETICAL

A semi-ideal model of chromatography was employed to model the separation of the thymine–thymine dimer from thymine. The mass balance equations for the ideal model were solved using numerical dispersion to account for the finite column efficiency[8].

The competitive Langmuir isotherm model was employed to relate the amount

adsorbed in the stationary phase at equilibrium to the concentration in the mobile phase. This isotherm provides a reasonable first approximation for most liquid–solid phase equilibria. The relationship giving the amount of component i sorbed at equilibrium with concentrations C_1 and C_2 of components 1 and 2, respectively, is

$$q_i = a_i C_i/(1 + b_1 C_1 + b_2 C_2) \tag{1}$$

Further simplifications to the competitive isotherm model were made in view of the nature of the problem. In this study, the concentration of the early eluting compound under the experimental conditions of interest, *i.e.*, the thymine–thymine dimer, is very low (between 1/1000 and 1/10 000). Hence the amount of sample injected is almost always insufficient for the dimer band to exhibit non-linear elution behavior. Therefore, it is not necessary to determine the coefficient b_1. As C_1 is very small, we can neglect the corresponding terms ($b_1 = 0$) and write as follows the competitive isotherm for the first component:

$$q_1 = a_1 C_1/(1 + b_2 C_2) \tag{2}$$

Similarly for the second component, the influence of such a small concentration of the dimer on the adsorption behavior of the monomer can be considered to be negligible, and therefore

$$q_2 = a_2 C_2/(1 + b_2 C_2) \tag{3}$$

Using these simplified isotherm models, the values of the coefficients a_i and b_i ($i = 1, 2$) are determined experimentally. From the retention time of a very small sample of thymine–thymine dimer, a_1 is determined, while a_2 and b_2 are derived from isotherm measurements made for thymine with the ECP method[4,6]. The isotherm data are then used to calculate the elution profiles of large samples of dilute solutions of thymine–thymine dimer in thymine.

RESULTS AND DISCUSSION

The elution chromatogram of a synthetic mixture of thymine monomer and thymine–thymine dimer is shown in Fig. 1. The retention time of the dimer is 1011 s and that of the monomer is 1101 s. The dead time, t_0, determined from the retention time of uracil is 195 s. The relative retention of the two components is 1.11, the resolution is 1.43 and the column efficiency is 5000 plates.

The adsorption equilibrium isotherm of thymine in the chromatographic system used was measured by the method of elution by characteristic point[6,7]. The data are reported in Fig. 2 by squares. The curvature of the isotherm is not very great within the range of concentrations in the mobile phase investigated, so the fit of the data on a Langmuir isotherm, as indicated by the solid line, is excellent.

The values of the coefficents obtained by a least-squares regression on eqn. 3 are $a_2 = 15.908$ ml/ml and $b_2 = 0.11774$ ml/mg. The coefficient a_1 is determined from the retention time of the pure dimer and is equal to 14.03 ml/ml.

Validity of the model

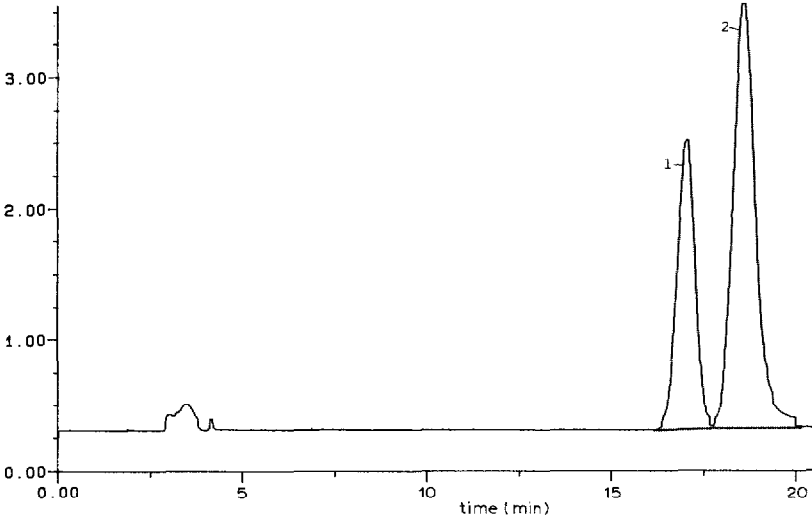Knowing the adsorption isotherm, the porosity, the mobile phase velocity and

Fig. 1. Experimental chromatogram of a 1:1 mixture of thymine–thymine dimer (band 1) and thymine (band 2). Column, 250 mm × 4.6 mm I.D., packed with 7-μm C_{18} bonded-phase silica; mobile phase, distilled water; flow-rate, 1 ml/min; sample size, 1.1 μg of each component; UV detection at 210 nm. Ordinate, detector signal in arbitrary units.
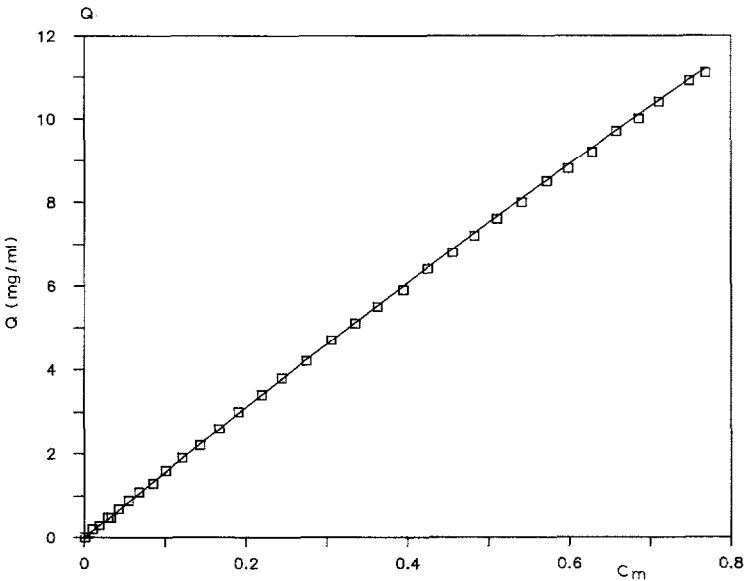

Fig. 2. Equilibrium isotherm of thymine obtained by the method of elution by characteristic point. Plot of the concentration of thymine adsorbed (Q) (mg/ml of column packing material) *versus* the concentration in the mobile phase at equilibrium (C_m) (mg/ml).
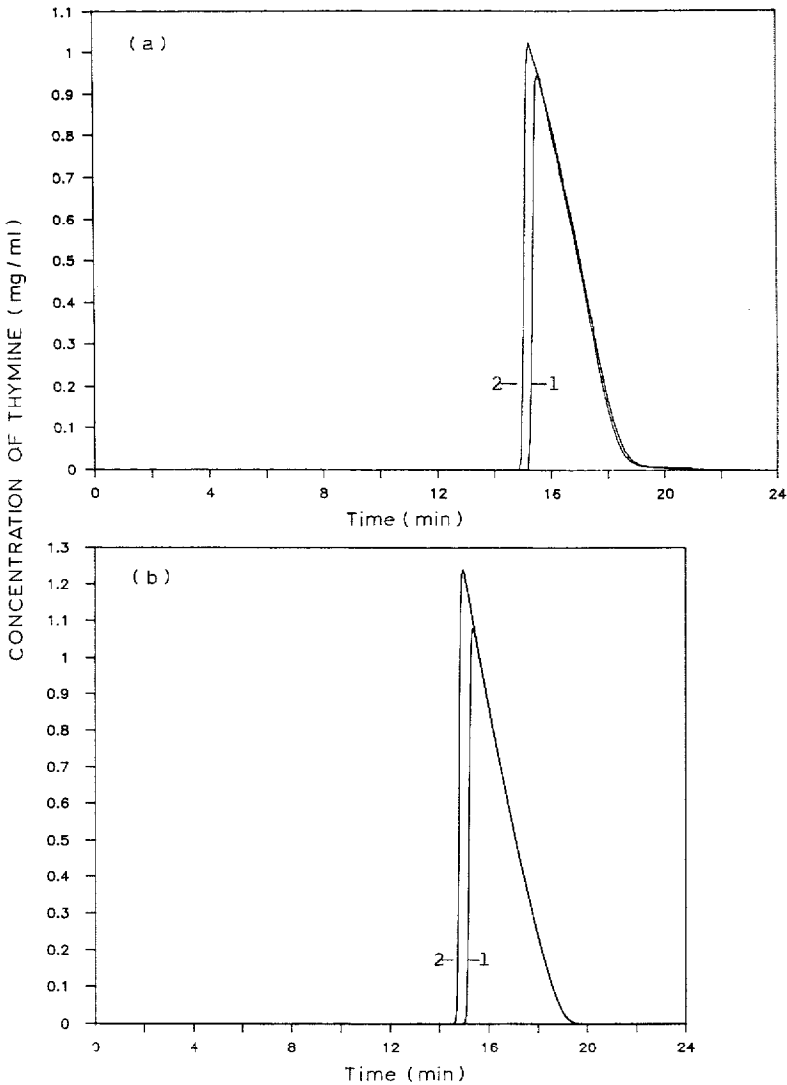
Fig. 3. Comparison between experimental and theoretical chromatograms. (a) Experimental chromatogram. Same conditions as in Fig. 1, except sample volume, (1) 1 ml and (2) 1.25 ml. Sample is a 2 mg/ml solution of thymine in water. (b) Predicted chromatograms based on the isotherm in Fig. 2, the measured efficiency and the sample size in (a).

the column dimensions, it is possible to make accurate predictions of the band profiles[3,4] of the thymine and the thymine–thymine dimer at different concentrations. Consider first the injection of a pure thymine solution at a concentration of 2 mg/ml. In Fig. 3a, curve 1 corresponds to a 1-ml injection and curve 2 to a 1.25-ml injection. The theoretically predicted profiles corresponding to the same experimental conditions are shown in Fig. 3b. They are in excellent agreement, except for a slight tail at the end of the elution band.
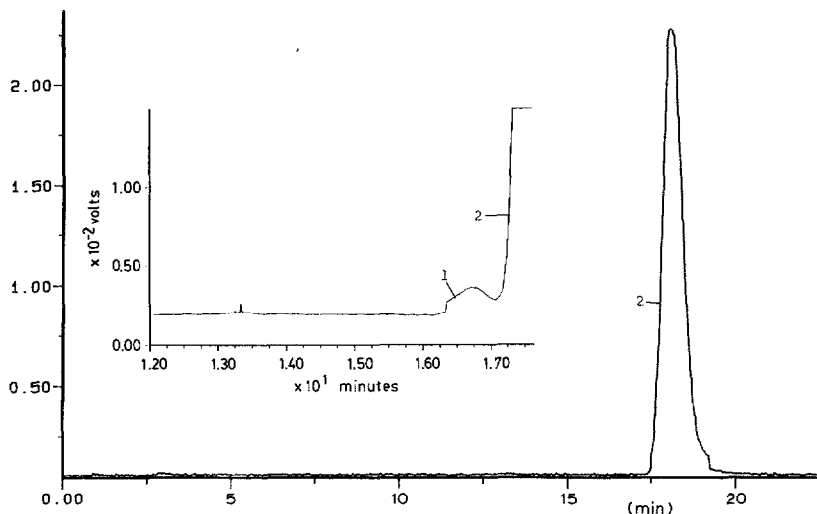
Fig. 4. Experimental chromatogram for a solution containing 82 ng of dimer (band 1) and 115 μg of thymine (band 2). The main figure shows the detector response at 300 nm (thymine) and the inset shows the detector response at 210 nm (dimer and thymine). Ordinates, detector response in volt.

This model also permits the prediction of the band profiles of binary mixtures. Fig. 4 shows an experimental uncalibrated band profile for a 1:1405 dimer–monomer mixture, recorded at 300 nm. The inset is an expansion of the dimer peak, recorded at 210 nm (see Experimental). Fig. 5 shows the predicted profile. The retention times of
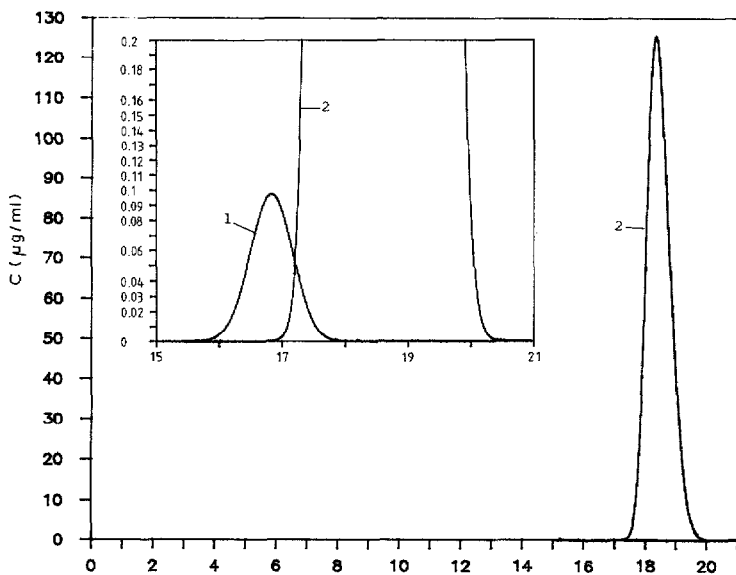

Fig. 5. Predicted chromatogram corresponding to the conditions in Fig. 4. The inset shows an expansion around the base of the thymine peak. 1, Elution profile of thymine; 2, elution profile of dimer. Ordinate: concentration (μg/ml); abscissa, time (min).

the peak maxima are the same for the theoretical and predicted profiles. Qualitatively, the peak shape of the experimental thymine band is very similar to the predicted band and the degree of resolution between the two bands is comparable. Similar results, not shown, were found for a 1:112 mixture.

Effect of sample size

The study of series of numerical solutions of the system of mass balance equations of the two compounds in the column (semi-ideal model[4]) allows the determination of the conditions of optimum operation. Fig. 6 shows the variation of the elution profile of 82 ng of dimer in samples containing increasing masses of thymine (0.082, 0.82, 2.0, 5.0 and 8.2 mg). This figure represents the calibrated detector response as if we were monitoring at a wavelength specific only to the dimer, totally excluding the thymine. Fig. 7 shows the elution profiles of thymine in the same samples, as if the calibrated detector response was now specific to thymine.

It can be seen from Figs. 6 and 7 that, on increasing the amount of thymine, the retention time of both compounds decreases and that the maximum concentration of the dimer band increases (Fig. 6). At the same time, the resolution between the two bands decreases. Eventually, at very large values of the sample size and/or at very low relative dimer concentrations, the small band of dimer is swallowed by the huge
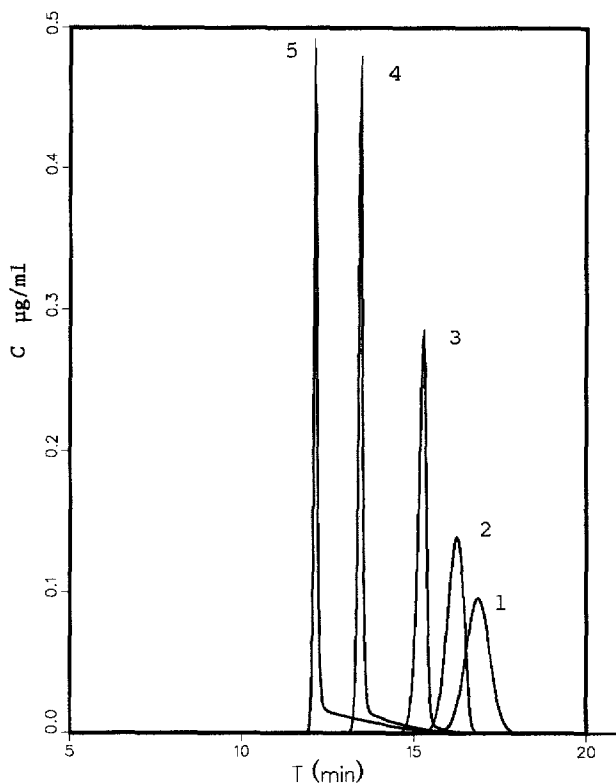

Fig. 6. Predicted dimer band profiles for samples containing 82 ng of the dimer in the presence of increasingly large amounts of thymine. Amount of thymine: 1, 0.082; 2, 0.82; 3, 2.5; 4, 5.0; 5, 8.2 mg.
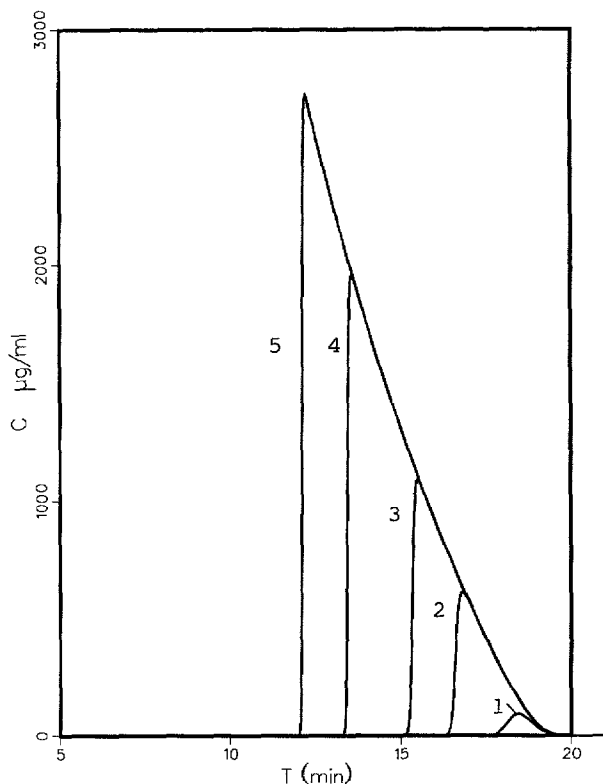
Fig. 7. Predicted thymine band profiles for samples containing increasingly large amounts of thymine in the presence of a constant amount (82 ng) of dimer. Amount of thymine: 1, 0.082; 2, 0.82; 3, 2.5; 4, 5.0; 5, 8.2 mg. Conditions as in Fig. 5.

monomer band (see Fig. 8). The same behavior is also seen when 8.2 or 0.82 ng of dimer is injected, only the maximum value of the ordinate is reduced to 0.05 and 0.005, respectively. The results in Fig. 6 are very important as they reveal a phenomenon often mentioned, but only casually, in the literature, regarding the displacement of the bands of minor or trace components by those of the main components when a column is overloaded.

The program also permits the determination of the optimum cutting time when the collection of the first band (the dimer) should be ended in order to achieve a recovery yield exceeding any given threshold. The lower the required value of the recovery yield, the larger is the proportion of the monomer that can be eliminated and the higher are the absolute and the relative concentrations of the dimer in the recovered fraction. Some typical results are given in Table I. A two-step collection scheme, with intermediate reinjection of the first collected fraction, has been attempted for very low values of the relative concentration of the dimer. The results are also given in Table I. All possible strategies of this sort could easily be simulated with the computer program, if needed, but our present needs were satisfied with the calculations just described.
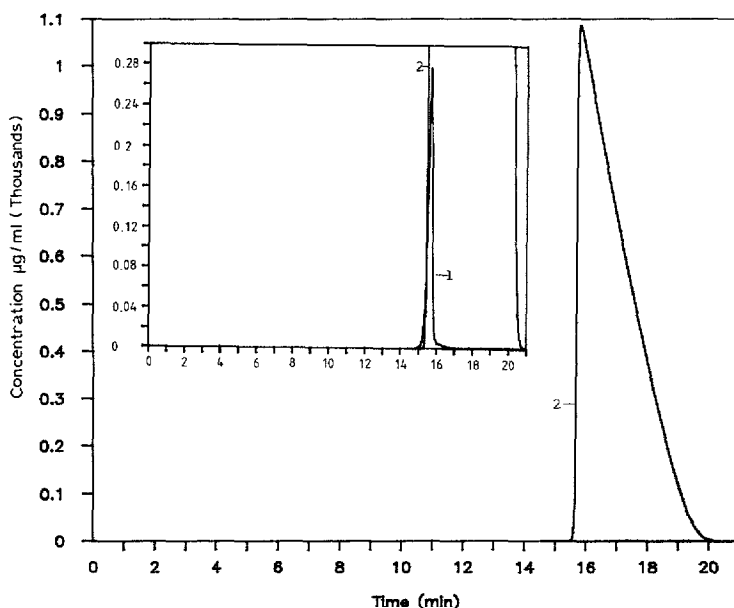
Fig. 8. Predicted band profiles for a sample of 82 ng of dimer (1) in the presence of 2 mg of thymine (2). The dimer band is shown in the inset. Conditions as in Fig. 5.

Enrichment simulation

The use of recycle techniques for the enrichment of traces of the dimer in the monomer was investigated numerically, which the program permits readily. The quantitative extraction of the dimer from 1:24 000 and 1:5000 (w/w) binary mixtures with the monomer was studied, using a sample containing 82 ng of dimer in the first instance and 400 ng of dimer in the second. This concentration range includes the estimate given above (0.02%) of the maximum concentration of structural alterations in DNA after physiologically significant radiation exposures[2].

TABLE I

ENRICHMENT BY RECYCLING

	Concentrations		
	Initial	*1st cycle[a]*	*2nd cycle[a]*
First case:			
Amount of dimer (ng)	82	78	74
Amount of thymine (mg)	2	0.13	$4.7 \cdot 10^{-5}$
Ratio (w/w)	1:24 300	1:1700	1:1.6
Second case:			
Amount of dimer (ng)	400	396	377.3
Amount of thymine (mg)	2	0.109	$3.45 \cdot 10^{-5}$
Ratio (w/w)	1:5000	1:290	10:1

[a] All yields are 95%.

Under these conditions the band is strongly overloaded, as can be observed in Fig. 8, showing the predicted elution bands for a 1:24 000 mixture containing 82 ng of dimer, and coelution of the two fronts takes place (see also Fig. 6, band 3). A fraction of the elution band was collected, so as to recover 95% of the early eluting component. This fraction contains a 1:1700 dimer–monomer mixture and its volume is between 1 and 2.5 ml, depending on the case. It was assumed that the volume of this fraction can be reduced to 100 μl and that it was reinjected, giving the chromatogram shown in Fig. 9. From this chromatogram, it was found that 95% of the dimer could be collected in a final fraction where it is considerably enriched to a ratio of 1:1.6.

A similar study was made with the 1:5000 mixture. After the first cycle, it was found that 95% of the early eluting compound could be recovered in a fraction containing a 1:287 (w/w) mixture. Note that substantial enrichment is obtained by accepting a slight decrease in yield. If a 99% yield was required, then only a 3-fold enrichment would occur, leaving a 1:1894 mixture. Subsequent reinjection of this first fraction led to a considerably enriched second fraction containing a 10:1 mixture.

Experimental study of enrichment

A comparison was made between the theoretical data in Figs. 8 and 9 and experimental results. Fig. 10 illustrates the elution chromatogram of 82 ng of dimer in the presence of 2 mg of thymine, monitored at 300 nm, with the experimental conditions as described above.

The retention times of the band front and of the rear boundary are in nearly quantitative agreement with Fig. 8. The slight difference in elution time is easily explained by the fact that the experiment was carried out several weeks after the
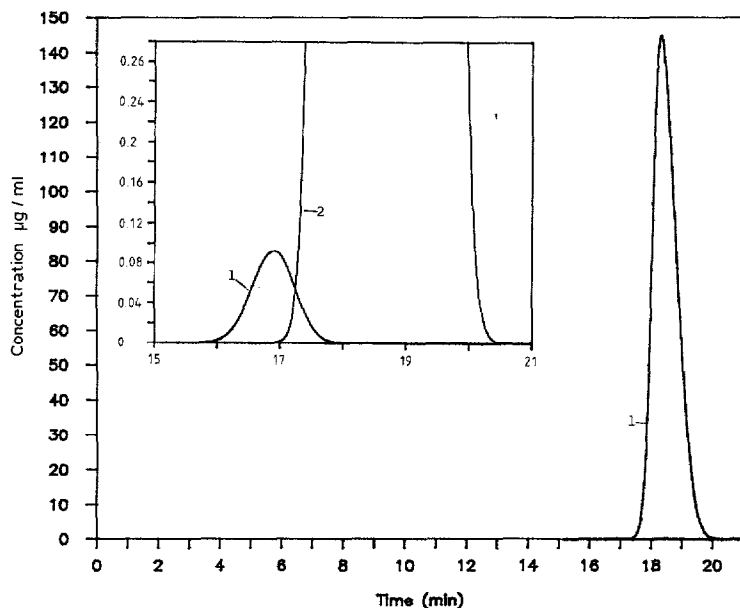

Fig. 9. Predicted band profiles for the first fraction collected (yield 95% for the dimer) for the chromatogram in Fig. 8. Conditions as in Fig. 5.
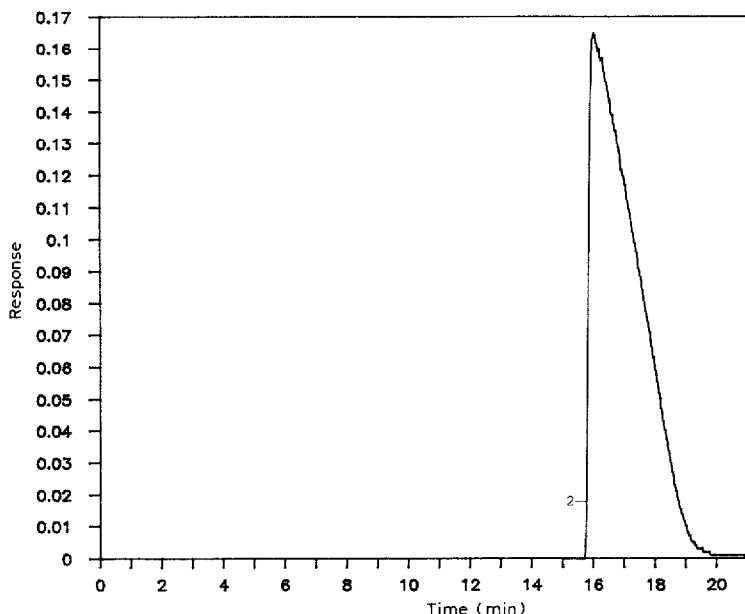
Fig. 10. Experimental band profile recorded with a UV detector at 300 nm for a sample of 82 ng of dimer in the presence of 2 mg of thymine. Only the thymine band (2) is visible. Experimental conditions as in Fig. 4.

determination of the isotherm. On collecting the eluate from 15.6 to 16.5 min, concentrating the fraction to 230 μl and reinjecting it under the same conditions, the chromatogram in Fig. 11 was recorded at 300 nm. The inset shows the chromatogram recorded with a detector setting at 210 nm.

The experimental chromatogram in Fig. 11 is in excellent agreement with the predicted result in Fig. 9. A good resolution is observed between the dimer and the thymine band, slightly better than predicted. The retention times of both bands are slightly shorter than predicted, which can be explained by the difficulty in reproducing the same flow-rate exactly. A more serious experimental problem prevents exact duplication of the conditions selected for the calculation runs, and also limits the reproducibility of these experimental results. The collection of fractions has to be made drop by drop, and the time when the last collected drop falls does not necessarily coincide with the optimum cutting time. As the amount of thymine recovered is very sensitive to the number of drops collected in the fraction, the composition of this fraction is different from that predicted. An experimental collection time slightly shorter than that used in the calculations explains the collection of smaller amounts of dimer and thymine, and hence a lower dimer yield and a better resolution between bands than predicted.

Hence overloaded elution permits the collection of highly enriched fractions of an impurity for their further quantitative analysis by other techniques that provide lower detection levels and better accuracy and precision, or possibly for investigation of their structure by sophisticated spectroscopic techniques, permitting the formal identification of molecules at extremely low sample sizes, but requiring pure samples.
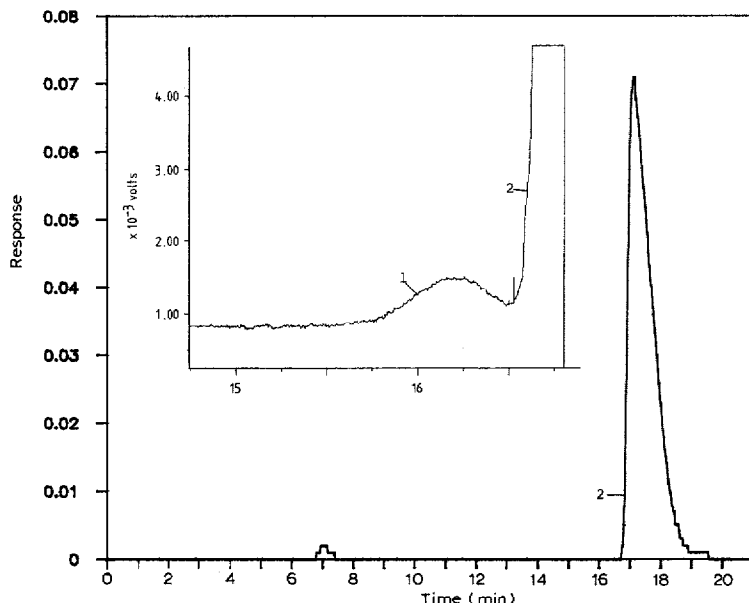
Fig. 11. Experimental band profiles for the first fraction collected during the analysis shown in Fig. 10. Main figure, UV detector signal set at 300 nm; inset, UV detector signal set at 210 nm. Experimental conditions as in Fig. 4.

The agreement between experimental results and the prediction of the semi-ideal model confirms the validity of the theory of non-linear chromatography previously developed[3,4].

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