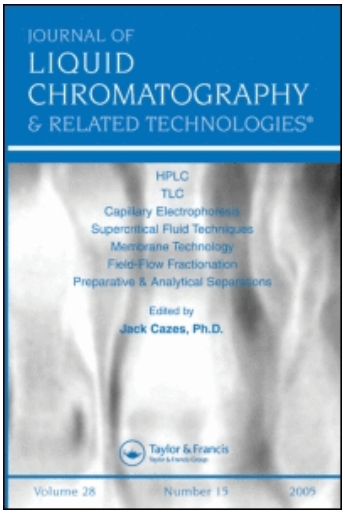

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Effect of Temperature and Mobile Phase Composition on RP-HPLC Separation of Cephalosporins

J. Martín-Villacorta[a]; R. Méndez[a]
[a] Departamento de Bioquímica y Biología Molecular Facultades de Biología y Veterinaria, Universidad de León, León, Spain

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EFFECT OF TEMPERATURE AND MOBILE PHASE COMPOSITION ON RP-HPLC SEPARATION OF CEPHALOSPORINS

J. MARTÍN-VILLACORTA* AND R. MÉNDEZ
Departamento de Bioquímica y Biología Molecular
Facultades de Biología y Veterinaria
Universidad de León
24071 León, Spain

ABSTRACT

The effects of mobile phase composition and column temperature on resolution in reversed-phase high performance liquid chromatography (RP-HPLC) were used to separate the following cephalosporins: cefonicid, cefaclor, cephazolin, cefodizime, cephaloridine, cephamandole and cephalotin, from a single sample. The capacity factor (k') was described as a function of temperature and mobile phase composition. Semi-empirically estimated values of k' were determined using a small number of experimental data for different temperatures between 20 °C and 60 °C, and mobile phase compositions (acetate buffer/isopropanol). The capacity factor of each cephalosporin was observed to decrease with increasing temperature and the volume fraction (V) of isopropanol in the mobile phase; linear relationships were obtained for plots of ln k' *versus* 1/T and log k' *versus* log V. The method developed, while simple, reveals the optimal isocratic elution conditions for column temperature and eluent concentration for the complete separation and rapid analysis of the cephalosporins studied.

*Correspondence

INTRODUCTION

In a previous paper (1) we reported the effect of column temperature on resolution in RP-HPLC to separate various penicillins from a single sample. Now we describe the effect of column temperature and volume fraction of an organic solvent on resolution in the isocratic elution conditions of some cephalosporins (Table 1).

The mobile phase composition and column temperature are two important experimental conditions that can be altered when a mixture of several compounds is separated (2-4).

In this paper, the relationships between capacity factor k' and organic modifier concentration in the mobile phase, and the effect of the column temperature on k' for the cephalosporins studied have been used to define k' as a function of T and V (volume fraction), on the basis of small number of experimental measurements for a given combination of column, organic solvent and cephalosporin. From calculated values of k' resolution values R_S may be estimated for adjacent band-pairs in all conditions. The method developed enables the optimization of RP-HPLC separations of the cephalosporins in the absence of hard theoretical calculations using a small number of experimental data, including the influence of the organic solvent in the mobile phase (isopropanol) and column temperature.

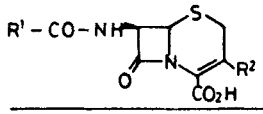
MATERIALS AND METHODS

Materials.

The cephalosporins were obtained from commercial products available for clinical use, except the cefodizime, which was supplied by Hoechst (Germany). Their structures are shown in table 1.

TABLE 1

Structures of cephalosporins



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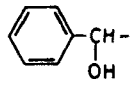
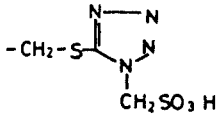
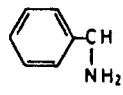
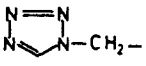
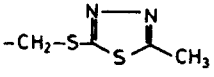
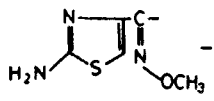
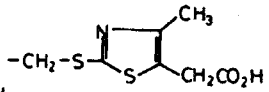
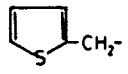
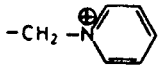
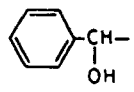
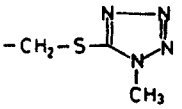
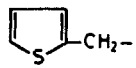
		1: Cefonicid
	$-Cl$	2: Cefaclor
		3: Cephazolin
		4: Cefodizime
		5: Cephaloridine
		6: Cephamandole
	$-CH_2-O-COCH_3$	7: Cephalotin

HPLC-grade water and isopropanol (Carlo Erba, Milan, Italy) were used in this study. All other chemicals (analytical grade) were obtained from Merck (Darmstadt, F.R.G.)

Instruments.

The HPLC system from Waters Assoc. consisted of a Model 600E multisolvent delivery system equipped with a heated column compartment, a Model 484 variable-wavelength detector, and a Model 745B computing integrator. The chromatograph was equipped with a Spherisorb ODS column (10 μm particle size, 25 cm x 4.6 mm I.D.).

Chromatographic Procedure.

The mobile phases used to separate the compounds were acetate buffer (pH 5.00, 0.1 M)/isopropanol, 96.5/3.5, 95/5, 93/7 and 90/10 (v/v). A pre-column (3 cm x 4.6 mm I.D.) packed with the same packing materials was used to guard the main column. The detector was set at 254 nm. The flow-rate of the mobile phase was 1.0 ml/min. The column dead time, t_o, was measured by injecting methanol.

RESULTS AND DISCUSSION

The mixture of cephalosporins was chromatographed at each of five column temperatures from 20 to 60 °C, and at four different volume fractions of isopropanol in mobile phase from 0.035 to 0.1. Figure 1 shows chromatograms with the volume fraction of isopropanol ranging from 0.035 to 0.1 at a constant column temperature (30 °C). Figure 2 shows the chromatograms obtained for each column temperature at 0.05 volume fraction of isopropanol. As one can see, a marked effect is produced for both parameters on the chromatographic behavior of each cephalosporin.

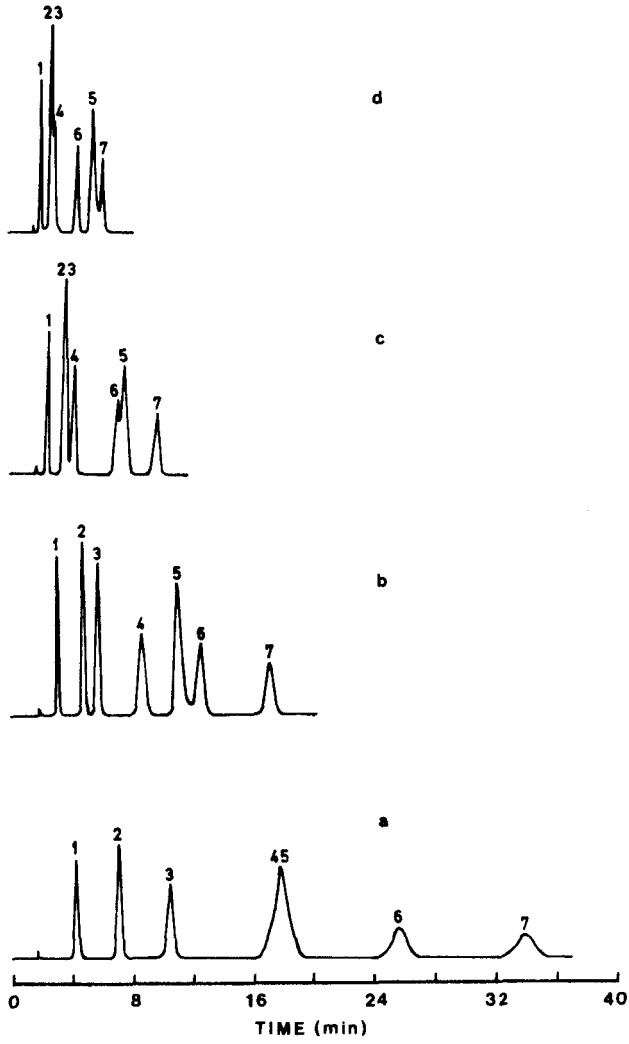


FIGURE 1. Effect of changing isopropanol volume fraction in mobile phase on the elution profiles of cefonicid (1), cefaclor (2), cephazolin (3), cefodizime (4), cephaloridine (5), cephamandole (6) and cephalotin (7), using a mobile phase of 0.1 M acetate buffer (pH 5.00)/isopropanol (v/v) a = (96.5/3.5), b = (95/5), c = (93/7) and d = (90/10) at column temperature of 30 °C.

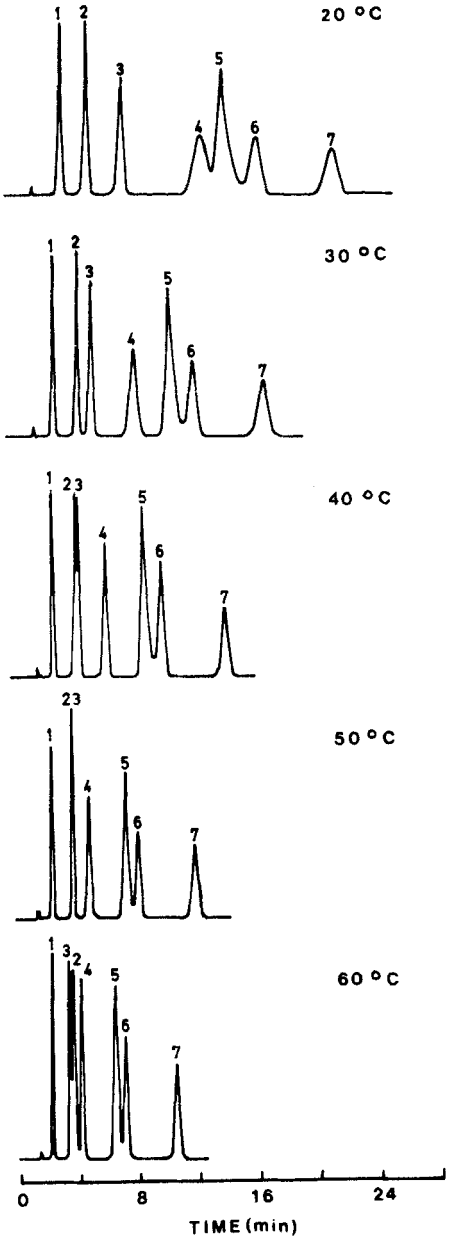


FIGURE 2. Effect of column temperature on the elution profiles of cephalosporins studied (numbering as in fig. 1), using a mobile phase of 0.1 M acetate buffer (pH 5.00)/isopropanol (95/5) (v/v).

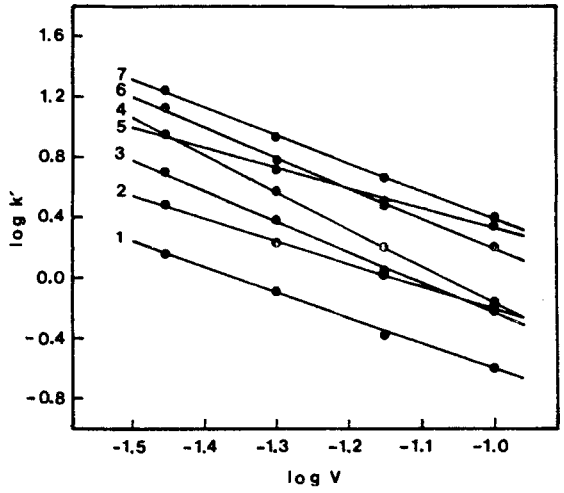


FIGURE 3. Effect of isopropanol volume fraction (V) in mobile phase on the capacity factor (k') at 30 °C. Mobile phases as in fig.2 and numbering as in fig. 1.

Capacity Factor as a Function of Volume Fraction of Isopropanol in Mobile Phase.

The volume fraction (V) of organic solvent in mobile phase is one of the most important parameters controlling capacity factors in RP-HPLC chromatography. Many reported studies (5-9) show that for a given solute and separation temperature T the relationship between capacity factor (k') and the volume fraction (V) or the eluent concentration can be expressed as follows.

$$k' = aV^{-b} \qquad \text{(Eq. 1)}$$

where a and b are constants.

For all cephalosporins the plots of log k' *versus* log V gave straight lines for all temperatures, with a correlation of 0.9992 (or more) (Fig. 3). As one

can see, there are different slopes, which may be ascribed to probably different separation mechanisms.

Table 2 lists constants a and b calculated from the intercepts and the slopes, respectively, by means of a least-squares method. The slopes for 1, 2 and 7 were found to be slightly affected by the column temperature, especially the slope for 1 (numbering as in table 1).

Capacity Factor as a Function of Column Temperature.

Figure 2 shows five chromatograms for the mixture of cephalosporins obtained at different temperatures. As one can see the retention time of each cephalosporin increased strongly as the column temperature increased from 20 to 60 °C.

The dependence of the capacity factor on temperature is given by the Van't Hoff equation:

$$\ln k' = -\Delta H^{o}/RT + \Delta S^{o}/R + \Phi \qquad \text{(Eq. 2)}$$

were R is the gas constant, ΔH^{o} and ΔS^{o} are the enthalpy and entropy changes, respectively, associated with the solute retention process. The parameter Φ is the phase ratio and T is the absolute temperature.

Figure 4 shows a Van't Hoff plot for each cephalosporin. It can be seen that the lines generated from the ln k' of each compound at the different temperatures are straight lines with a correlation coefficient of 0.995 (or more). The linearity of the plots supports the assumption that single sorption mechanisms are operative for each cephalosporin. As for penicillins and other compounds (1,10,11), the slopes of these lines were not all equal, as one might expect if the effect of temperature was generalized.

For all cephalosporins the values of enthalpy change are negative (table 3), which indicates that the transfer of cephalosporin from the mobile

TABLE 2

Values of Constants a and b in Equation 1 at Different Column Temperatures

Cephalosporins	Column temperature (°C)									
	20		30		40		50		60	
	b	a x 10^3	b	a x 10^3	b	a x 10^3	b	a x 10^3	b	a x 10^3
1	1.43	13.9	1.67	5.07	1.82	2.48	2.18	0.665	2.24	0.472
2	1.41	29.5	1.49	20.0	1.55	14.1	1.52	13.4	1.65	7.86
3	2.01	7.86	2.03	5.23	1.99	4.23	2.20	1.71	2.14	1.60
4	2.50	3.22	2.46	2.29	2.48	1.54	2.71	0.559	2.70	0.410
5	1.24	163.7	1.35	92.6	1.36	68.7	1.33	60.3	1.42	37.6
6	1.96	22.8	2.03	14.1	2.03	10.4	2.04	8.25	2.20	4.30
7	1.74	59.5	1.86	33.0	1.87	25.9	2.02	13.7	1.99	12.6

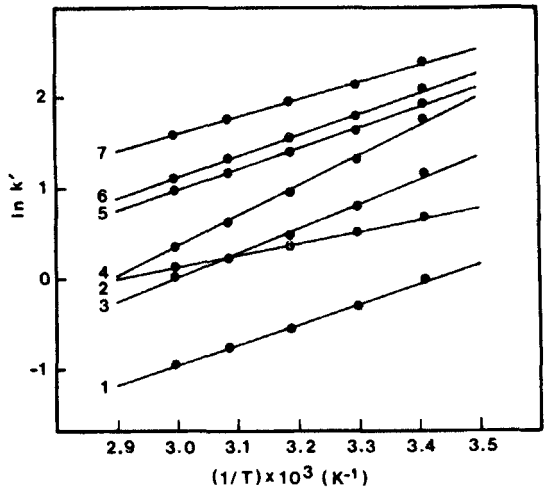


FIGURE 4. Effect of column temperature on the capacity factor (k'). Mobile phase as in fig. 2 and numbering as in fig. 1.

TABLE 3

Values of ΔH^{o} at Various Volume Fractions of Isopropanol in Mobile Phase. Numbering as in Table 1.

Cephalosporin	Volume Fraction of Isopropanol			
	0.035	0.05	0.07	0.10
	ΔH^{o} (kJ mol^{-1})	ΔH^{o} (kJ mol^{-1})	ΔH^{o} (kJ mol^{-1})	ΔH^{o} (kJ mol^{-1})
1	-14.1	-19.2	-23.5	-32.6
2	-11.0	-11.7	-12.3	-15.6
3	-24.0	-24.1	-24.6	-27.6
4	-26.6	-29.7	-30.9	-32.2
5	-18.9	-19.4	-19.4	-21.9
6	-17.7	-20.0	-20.4	-22.1
7	-14.8	-16.1	-17.8	-20.4

phase to sorption sites is favored. The enthalpy change values are similar to those of penicillins (1).

Determination of Capacity Factor for any Value of Volume Fraction of Isopropanol and Column Temperature.

Following the methodology developed by Gant and coworkers (2) it is possible to obtain the values for the capacity factor ($k'_{T,V}$) for any value of volume fraction of isopropanol and column temperature. In this methodology a standard state is defined by a temperature (T_S) and mobile phase composition (V_S). In the present study, T_S = 20 °C (293.3 K) and V_S = 0.035. The standard state value of k' for the solute in question is k'_{T_S,V_S}.

From equation 1 we can write:

$$\log k'_{T_S,V} = \log k'_{T_S,V_S} - b(\log V - \log V_S) \qquad \text{(Eq. 3)}$$

where $k'_{T_S,V}$ is the capacity factor for a value of V and the temperature T_S.

From equation 2 we can write at any value of T and V.

$$\log k'_{T,V} = \log k'_{T_S,V} - c(1/T_S - 1/T) \qquad \text{(Eq. 4)}$$

where $k'_{T,V}$ is the capacity factor for any value of T and V, and the parameter c varies with the cephalosporin and with mobile phase composition.

According to equation 3 and 4, the temperature coefficient c must be of the form

$$c = d - e \log V \qquad \text{(Eq. 5)}$$

where d and e are constant for a given cephalosporin and system.

Using equations 3, 4 and 5 it is possible to calculate the capacity factor $k_{T,V}$ for any value of T and V, after determining the value of b and c from these equations and the experimental data. The parameter c used for each volume fraction of isopropanol is determined from equation 5, using the

TABLE 4

Experimental *versus* Calculated Capacity Factor (k') Values for Cephalosporins Studied at Different Temperatures. Volume Fraction of Isopropanol V = 0.035. Numbering as in Table 1.

Cephalosporin	Temperature (°C)									
	20		30		40		50		60	
	Exp.	Calc.	Exp.	Calc.	Exp.	Calc.	Exp.	Calc.	Exp.	Calc.
1	1.69	1.69	1.43	1.43	1.15	1.22	1.00	1.03	0.85	0.88
2	3.36	3.36	3.00	2.95	2.55	2.59	2.18	2.28	2.00	2.00
3	6.84	6.84	4.92	5.13	3.45	3.85	2.80	2.88	2.07	2.16
4	13.5	13.5	8.80	9.64	6.30	6.89	4.90	4.92	3.48	3.51
5	10.5	10.5	8.80	8.40	6.54	6.67	5.15	5.30	4.27	4.21
6	16.2	16.2	13.1	13.0	9.65	10.4	8.00	8.31	6.96	6.65
7	20.4	20.4	17.6	17.0	14.1	14.2	12.0	11.9	9.82	9.94

TABLE 5

Experimental *versus* Calculated Capacity Factor (k') Values for Cephalosporins Studied at Different Temperatures. Volume Fraction of Isopropanol V = 0.05. Numbering as in Table 1.

Cephalosporin	Temperature (°C)									
	20		30		40		50		60	
	Exp.	Calc.	Exp.	Calc.	Exp.	Calc.	Exp.	Calc.	Exp.	Calc.
1	1.01	1.01	0.75	0.79	0.58	0.62	0.47	0.49	0.39	0.38
2	2.03	2.03	1.69	1.75	1.46	1.51	1.26	1.30	1.14	1.12
3	3.36	3.33	2.23	2.46	1.63	1.82	1.26	1.34	1.01	0.99
4	6.31	5.52	3.75	3.85	2.60	2.69	1.87	1.88	1.42	1.31
5	7.03	6.78	5.21	5.33	4.00	4.18	3.23	3.29	2.69	2.58
6	8.34	8.05	6.03	6.33	4.66	4.98	3.73	3.92	3.08	3.08
7	11.1	10.9	8.58	8.94	7.00	7.30	5.85	5.96	4.98	4.86

TABLE 6

Experimental *versus* Calculated Capacity Factor (k') Values for Cephalosporins Studied at Different Temperatures. Volume Fraction of Isopropanol V = 0.07. Numbering as in Table 1.

Cephalosporin	Temperature (°C)									
	20		30		40		50		60	
	Exp.	Calc.	Exp.	Calc.	Exp.	Calc.	Exp.	Calc.	Exp.	Calc.
1	0.61	0.63	0.41	0.46	0.31	0.34	0.23	0.25	0.19	0.18
2	1.23	1.26	1.02	1.07	0.87	0.90	0.77	0.77	0.66	0.65
3	1.60	1.69	1.09	1.23	0.81	0.89	0.58	0.65	0.48	0.47
4	2.51	2.38	1.58	1.62	1.17	1.11	0.79	0.76	0.52	0.52
5	4.41	4.45	3.18	3.46	2.50	2.69	2.10	2.09	1.64	1.62
6	4.11	4.16	2.99	3.22	2.27	2.49	1.84	1.93	1.48	1.49
7	6.05	6.10	4.49	4.87	3.68	3.89	2.90	3.10	2.51	2.48

TABLE 7

Experimental *versus* Calculated Capacity Factor (k') Values for Cephalosporins Studied at Different Temperatures. Volume Fraction of Isopropanol V = 0.10. Numbering as in Table 1.

Cephalosporin	Temperature (°C)									
	20		30		40		50		60	
	Exp.	Calc.	Exp.	Calc.	Exp.	Calc.	Exp.	Calc.	Exp.	Calc.
1	0.38	0.38	0.25	0.26	0.17	0.17	0.10	0.12	0.08	0.08
2	0.77	0.76	0.63	0.63	0.50	0.52	0.44	0.44	0.35	0.36
3	0.84	0.82	0.59	0.59	0.43	0.42	0.28	0.30	0.22	0.22
4	1.00	0.97	0.67	0.65	0.46	0.43	0.28	0.29	0.21	0.19
5	2.90	2.86	2.16	2.20	1.58	1.69	1.27	1.30	0.97	1.00
6	2.10	2.07	1.57	1.57	1.15	1.19	0.94	0.91	0.69	0.69
7	3.30	3.27	2.50	2.55	1.98	1.99	1.45	1.55	1.22	1.21

constants d and e, previously determined by plotting experimental c values *versus* log V. Tables 4 - 7 compare experimental and calculated values of the capacity factor, k', for the cephalosporins studied, experimental k' values being predicted generally with good agreement.

Effect of Elution Conditions on Resolution.

The conventional equation to evaluate the effect of elution conditions on resolution (R_S) is :

$$R_S = 1/4\sqrt{N}\ (k'/1+k')\ (\alpha-1/\alpha) \qquad \text{(Eq. 6)}$$

Here N is the plate number, α is the selectivity factor (defined as k'_2/k'_1) were k'_1 and k'_2 are the capacity factors for band 1 and 2, and k' is the average capacity factor of k'_1 and k'_2. The three factors, α , N and k' control the resolution. It is assumed that the three terms of equation 6 are approximately independent, which allows their separate optimization. The resolution equation (Eq. 6) must be applied to each of the adjacent band-pairs considered.

As one can see in fig. 2 the band broadening of any peak appearing at a fixed retention time decreases as the temperature increases; accordingly, the N value increases with increasing temperature. A linear relationship was obtained for the plot of N *versus* T : N = 24.7 T - 6560 (T has the dimension of absolute temperature) with a correlation coefficient of 0.991 (fig. 5). The N value is practically independent of the volume fraction of isopropanol for the cephalosporins studied.

In general, the contribution of $k'/1+k'$ terms to Rs is approximately constant as long as k' is not small. Since the selectivity factor (α) is of greater concern in the equation 6, the separate optimization of the influence of the temperature and mobile phase composition in values is in

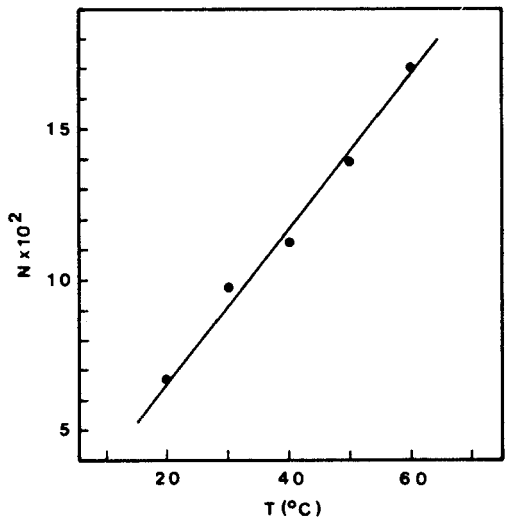


FIGURE 5. Effect of column temperature on plate number (N) for the analysis of the cephalosporins studied.

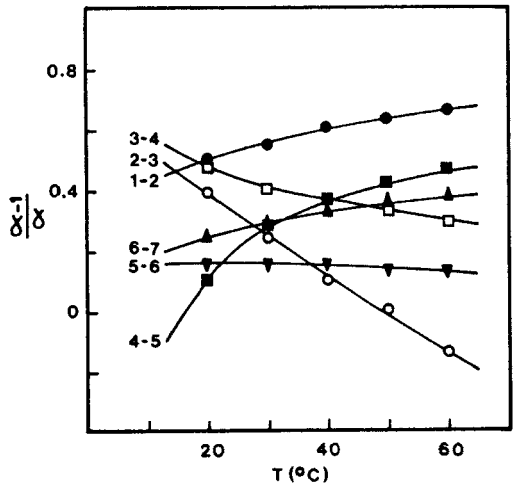


FIGURE 6. Effect of column temperature on the selectivity factor (α-1/α) of the six pairs of sequentially resolved peaks. Mobile phase as in fig. 2 and numbering as in fig. 1.

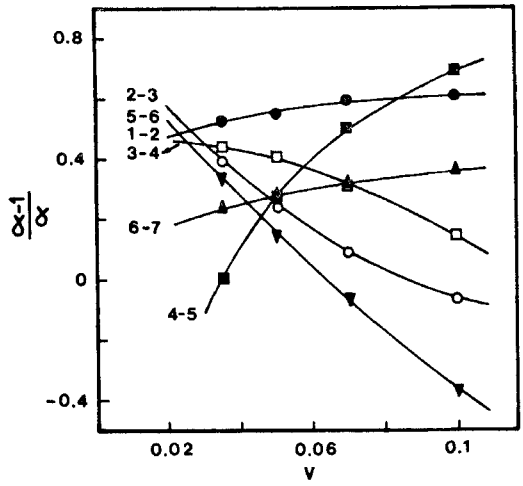


FIGURE 7. Effect of isopropanol volume fraction (V) in mobile phase on the selectivity factor (α-1/α) of the six pairs of sequentially resolved peaks at 30 °C. Mobile phases and numbering as in fig. 1.

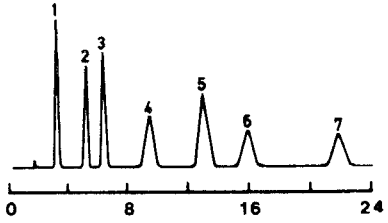


FIGURE 8. Isocratic elution profile under optimal conditions. Mobile phase 0.1 M acetate buffer (pH 5.00)/isopropanol (95.4/4.6) (v/v), Column temperature, 32 °C, numbering as in fig. 1.

many cases a good criterion for establishing the elution conditions of the separation.

Figures 6 and 7 show the influence of temperature and isopropanol volume fraction in the term $(\alpha-1/\alpha)$. There are examples of different situations: for pairs 5-6 and 4-5 the $(\alpha-1/\alpha)$ term is markedly influenced by the isopropanol volume fraction; the column temperature also exerts an important influence on this term for pairs 2-3 and 4-5. It is interesting to note that both parameters (T and V) have important and similar effects on pairs 4-5 and 2-3 in this resolution term. For these cases, both parameters can be used to obtain a better resolution.

CONCLUSION

The present study demostrates that it is possible with a small number of initial experimental data of the capacity factor (k') to predict k' for each cephalosporin as a function of T and V, which reveals the optimal elution conditions for the isocratic separation of a mixture of cephalosporins. Figure 8 shows an elution profile under optimal elution conditions.

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