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INFLUENCE OF TEMPERATURE IN REVERSE-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH GRADIENT ELUTION

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SUMMARY

The influence of temperature on the retention of several species separated by reverse-phase liquid chromatography by gradient elution is shown to be of enough importance to warrant careful control of temperature if reproducible results are to be obtained. The smaller the particle size in the column, the greater the effect of temperature, and therefore the control should be greater. Likewise, it has been verified that for a given solvent gradient, independent of its complexity, there is a linear relation between ln k' and $1/T$, which also occurs in separations by isocratic elution. Dufek's equation can be adjusted perfectly to the experimental data obtained from gradient elutions, and may be used in the simulation and optimization of gradient chromatographic processes.

In order to improve the efficiency of reverse-phase high-performance liquid chromatography (HPLC), it is necessary to consider the combined effect of the composition of the mobile phase and the column temperature on solute retention. The effect of varying the composition of the mobile phase is extremely helpful for the separation of complex mixtures. The use of more or less complicated gradients allows the resolution of peaks to be improved. Likewise, by controlling the temperature factor in such elutions, the reproducibility and selectivity of the chromatographic process can be improved.

The influence of temperature on solute retention in reverse-phase HPLC has mainly been studied in isocratic solutions [1—7]. In most of these studies, however, homologous series have been used to establish a correlation between retention and the number of carbon atoms in the solute molecule for each particular series. These studies agree that the relationship between the logarithm of the capacity factor (k') and the reciprocal of absolute temperature is linear, even over wide temperature intervals. Melander et al. [1—3] proposed an extrathermodynamic equation and a thermodynamic model [3] supporting the former, to explain the observed relationship between retention and temperature. This equation has also been verified by Vigh and Varga-Puchony [4]. Other authors [6, 7] have established another series of equations which relate temperature to the composition of the mobile phase, and the number of carbon atoms in the molecules of the homologous series.

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In theory, all these equations could not be applicable to gradient elutions, as the composition of the mobile phase varies during elution and this parameter is considered to be a constant in all the equations proposed. However, these models may be extended to gradient elutions, assuming that these behave similarly to isocratic elutions, if the same gradient is used in the study of the influence of temperature. This would allow the temperature factor readily to be included into the optimization of gradient elutions.

Unfortunately, no data are available in the literature concerning the extent of the influence of temperature on solute retention in gradient elutions, although it is almost certainly important. In practice, gradient elutions are used when the objective is to decrease separation time and increase efficiency, or when the solute mixture is too complex to be dealt with by isocratic separation. The variation in solute retention times owing to variations in temperature may provoke the cross-over or overlapping of peaks, thus invalidating laboriously developed methods. These problems are more likely to appear with more complex gradients (e.g., stepwise gradients). Common practice is to work at constant temperature to avoid such problems. Nevertheless, it is necessary to choose the temperature carefully, depending on the species to be separated. It is clear that if variation in retention times of species in gradient elutions is linear with temperature, the choice will be made considerably easier, as any of the optimization techniques proposed in the literature may be used [8]. Likewise, it is interesting to establish up to which point such an influence depends on the type of column or particle size, and therefore, up to which point the optimization of this parameter is critical for any particular working conditions. In the present paper, the influence of temperature on the gradient elution of 22 polyphenolic acids has been studied for two column types with different particle sizes.

EXPERIMENTAL

Waters HPLC equipment was used, comprising two pumps (M6000A and M45), a universal injector UK-6, a gradient programmer M680, and an M440 double-channel detector with absorbance filters of 280 and 340 nm, an Omniscribe printer and a Perkin-Elmer Sigma-15 data station. Two types of columns were used: a Waters μBondapak C18 (30 cm × 3.9 mm, particle size 10 μm) and a Waters Novapak C18 (15 cm × 3.9 mm, particle size 5 μm).

The polyphenolic acids used were from Fluka, Eastman and Merck (all >97% purity). These acids were dissolved in methanol/water mixtures according to their solubility) in concentrations ranging from 4 to 60 μg ml^{-1}, so as to obtain adequate responses from the detector. The solvents used (HPLC grade) were filtered through 0.45-μm filters (Millipore) and degassed for 30 min in an ultrasonic bath (Selecta).

The columns and solvents were thermostatted in a bath with a Selecta cryostat. The chromatographic conditions were as follows. The mobile phase

TABLE 1

Elution programs[a]

Novapak column				μBondapak column			
Time (min)	% A	% B	Curve	Time (min)	% A	% B	Curve
0.00	100	0	—	0.00	100	0	—
9.38	100	0	1	5.00	100	0	9
22.78	87	13	1	16.33	70	30	9
37.00	75	25	1	24.33	70	30	1
49.66	62	38	1	25.88	50	50	9
59.39	50	50	1				

[a]For A and B, see text.

was solvent A [2% acetic acid, 10% methanol in water (v/v)] or solvent B [2% acetic acid, 90% methanol in water (v/v)]. The solvent flow-rate was 1 ml min^{-1}, and the detection sensitivity, 0.05 absorbance full scale. The elution programs are given in Table 1.

RESULTS AND DISCUSSION

The starting point for this study lay in the observation of variations in the retention times of a group of 22 phenolic acids, for which a plan for gradient separation had empirically been drawn up as described in Table 1 (μBondapak C18 column) [9]. This gradient had been used routinely in a non-thermostatted system. Variations in the retention times, which sometimes provoked changes in selectivity giving rise to the overlapping of species which obviously hindered their correct quantitation, had often been observed in the non-thermostatted system. It was also decided that a more efficient column of smaller particle size (Novapak) should be tried, so as to improve the separation of these 22 species.

Attempts to use this second procedure routinely gave much greater variations in retention times than those obtained with the former column, even to such an extent that 25—30% of the species in the mixture could not reliably be quantified. After making allowance for the variability which could be put down to other parameters (faults in the instrumental system, variations in the composition of the mobile phases, etc.), it was decided that the effects of temperature on separation should be studied. For this, the mixture of 22 species was eluted with the gradients developed for each of the two columns, within 14—29°C, at intervals of 2°C. In cases where peak overlapping was detected, the mixture was split into simpler mixtures in order to obtain accurate retention times. Retention-time data obtained for each species and temperature were transformed to $\ln k'$ values and least-squares plots vs. the reciprocal of absolute temperature were obtained. The results of these calculations for both columns are included in Table 2 and shown in Fig. 1. Some interesting features may be observed.

TABLE 2

Correlation coefficients (r^2), slopes and intercepts of plots in ln k' vs. $(1/T) \times 10^{-3}$ for the elution of phenolic acids from a μBondapak C18 column and a Novapak column

No.	Acid	μBondapak column			Novapak column		
		r^2[a]	Slope	Intercept	r^2[a]	Slope	Inte
1	3,4,5-Trihydroxybenzoic	0.9974	2.70	−9.07	0.9944	3.34	−11.68
2	2,4,6-Trihydroxybenzoic	0.9977	3.03	−9.38	0.9864	5.33	−18.19
3	2,4-Dihydroxybenzoic	0.9941	2.45	−7.32	0.9982	2.99	−9.43
4	3,5-Dihydroxybenzoic	0.9972	2.69	−8.08	0.9940	3.53	−11.27
5	2,5-Dihydroxybenzoic	0.9931	2.32	−6.26	0.9983	3.12	−9.22
6	4-Hydroxybenzoic	0.9930	1.92	−4.84	0.9985	2.84	−8.18
7	2,6-Dihydroxybenzoic	0.9995	1.45	−3.18	0.9981	3.20	−10.02
8	3-Hydroxybenzoic	0.9958	0.99	−1.52	0.9970	2.85	−7.81
9	2,4-Dihydroxybenzoic	0.9988	0.95	−1.32	0.9986	3.26	−9.21
10	4-Hydroxy-3-methoxybenzoic	0.9925	0.67	−0.38	0.9967	2.85	−7.69
11	2,6-Dimethoxybenzoic	0.9798	0.40	0.58	0.9885	2.17	−4.95
12	3,4-Dihydroxycinnamic	0.9898	0.72	−0.49	0.9988	3.55	−9.55
13	3,5-Dimethoxy-4-hydroxybenzoic	0.9867	0.60	−0.63	0.9924	1.75	−3.55
14	4-Hydroxycinnamic	0.9905	1.03	−1.36	0.9959	1.95	−3.95
15	2,4-Dimethoxybenzoic	0.9915	0.50	−0.56	0.9954	1.64	−2.55
16	4-Hydroxy-3-methoxycinnamic	0.9902	0.90	−0.84	0.9942	1.74	−3.02
17	3-Hydroxycinnamic	0.9922	0.86	−0.68	0.9961	2.13	−4.34
18	3,4-Dimethoxybenzoic	0.9945	0.78	−0.43	0.9961	2.13	−4.34
19	2-Hydroxycinnamic	0.9920	0.43	0.83	0.9868	1.70	−2.87
20	3,5-Dimethoxy-4-hydroxycinnamic	0.9909	0.75	−0.32	0.9941	1.83	−3.18
21	3,5-Dimethoxybenzoic	0.9817	0.52	0.65	0.9897	1.37	−1.26
22	3,4,5-Trimethoxycinnamic	0.9992	0.38	1.10	0.9954	1.20	−0.60

[a] 5 points.

In all cases, irrespective of the type of column or gradient configuration, linear relationships are obtained between ln k' and $1/T$ (van't Hoff graphs), just as for isocratic elution. In general, the slope decreases with increasing affinity of the compound for the stationary phase, the decrease being greater for all species on the 5-μm column; important changes are produced in the order of elution of several compounds. Figure 1 shows the occurrence of several peak cross-overs between different species. For instance, peaks 3 and 4 show cross-over for the two types of column. While peak 3 hardly changes its behaviour as a function of column type, peak 4 shows a much greater variation in retention in the μBondapak column, so that it is possible, by using this column, to obtain a good resolution of this pair of peaks at temperatures higher than 23°C.

Some peaks show important behavioural differences as a function of column type (e.g., peak 15) but their behaviour as a function of the temperature remains very similar no matter which column is used. Although the temperature range considered is relatively small and corresponds approximately to a normal temperature range in laboratories that are not thermally conditioned,

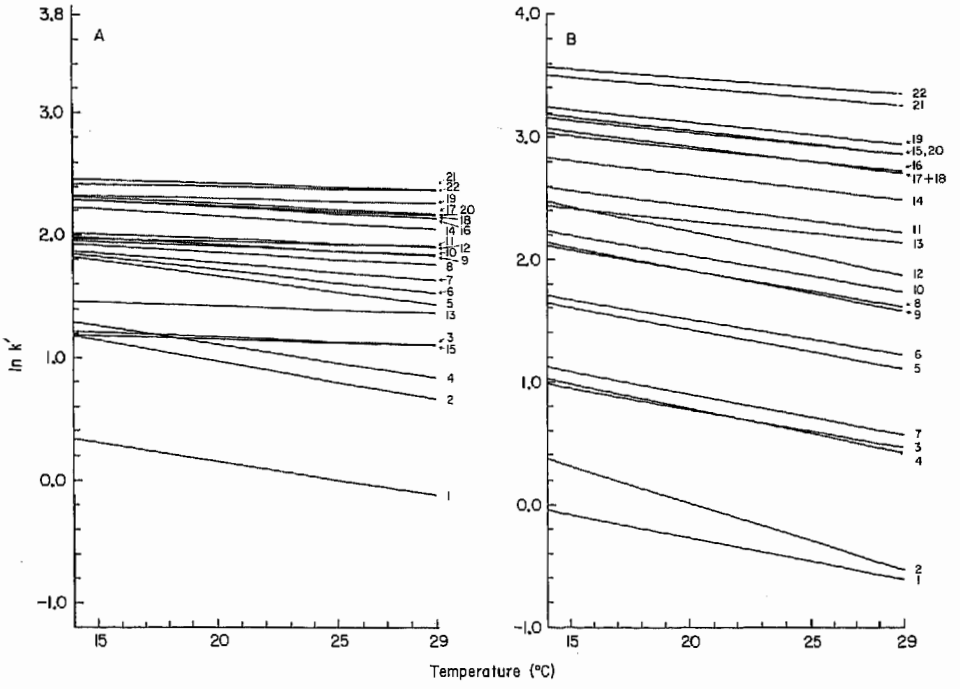

Fig. 1. Plots of ln k' vs. T for phenolic acids: (A) μBondapak column; (B) Novapak column. (See Table 2 for key to compounds.)

the effect on absolute and relative peak retention is considerable. Thus, several species change their retention times by more than 5 min over the temperature interval considered. This can be seen in Table 3, where retention time data for each species in the temperature range considered, as well as the absolute variation and the percentage variation of k' in this range (referred to the maximum value of k' obtained in each case) are collected for the two types of column. Given that the influence of temperature is much greater when 5-μm columns are used, a much more careful control of temperature will be needed in such cases.

The linearity of the relationship might have important consequences from the point of view of the optimization of gradient separations, given that once the most appropriate gradient program has been chosen for the resolution of a mixture, three or four experiments at different temperatures may help to establish the optimum working temperature with that particular gradient and the temperature margin in which the gradient will give satisfactory results. This will help to decide the temperature variation allowed in each case. Temperature could also be included in sequential optimization schemes based on simulation methods, taking advantage of this linear relationship.

The above linear variation of retention with reciprocal temperature in

TABLE 3

Values of retention time and increases in k' as a function of temperature and column[a]

Acid	μ-Bondapak				Novapak			
	t_R (min)		$\Delta k'$	$\Delta k'$ (%)	t_R (min)		$\Delta k'$	$\Delta k'$ (%)
	15°C	29°C			14°C	29°C		
1	7.51	5.91	0.50	36.2	2.40	1.88	0.42	44.2
2	13.25	9.39	1.23	38.3	2.94	1.88	0.86	61.8
3	13.51	10.04	1.10	33.4	4.41	3.05	1.14	48.5
4	14.47	10.48	1.26	35.1	4.63	3.15	1.27	44.9
5	22.15	16.11	1.92	31.8	7.61	4.96	2.20	42.1
6	22.62	17.25	1.70	27.5	8.09	5.43	2.25	39.7
7	23.43	19.14	1.36	21.1	5.06	3.43	1.34	42.8
8	24.88	21.53	1.07	15.5	11.35	7.26	3.43	41.2
9	25.68	22.58	0.98	13.7	11.82	7.49	3.63	41.6
10	25.37	22.96	0.76	10.8	12.76	8.28	3.83	40.1
11	25.68	24.35	0.42	5.9	17.39	12.25	4.36	32.7
12	26.51	23.89	0.84	11.3	15.85	11.62	3.53	29.5
13	27.04	24.83	0.70	9.2	15.50	9.23	5.23	44.6
14	32.41	27.80	1.46	15.7	22.77	16.52	5.14	29.2
15	34.71	32.11	0.83	8.3	29.75	22.67	5.80	24.8
16	33.81	29.79	1.27	13.0	26.96	19.89	5.84	27.8
17	34.24	30.33	1.24	12.6	28.47	19.89	7.04	31.7
18	33.47	29.85	1.15	11.9	28.47	19.89	7.04	31.7
19	35.66	33.43	0.71	6.9	22.29	24.34	6.5	25.7
20	34.10	30.69	1.09	11.1	30.52	22.67	6.42	26.8
21	40.07	37.05	0.96	8.2	43.37	33.96	7.67	22.4
22	38.73	36.57	0.69	6.1	44.33	36.60	6.31	18.0

[a]See Table 2 for identity of acids. t_R is retention time.

gradient elutions suggests that the equations developed for isocratic elution would be equally valid in this case. Amongst these equations, that of Dufek [7] is probably one of the simplest and most efficient for the calculation of capacity factors as a function of temperature:

$$\log k'_3 = \log k'_1 + \log (k'_2/k'_1)\,(1/T_3 - 1/T_1)/(1/T_2 - 1/T_1) =$$

$$\log k'_1 + \log (k'_2/k'_1)\,(T_2\ (T_1 - T_3)/T_3\ (T_1 - T_2))$$

where k'_1 and k'_2 are the capacity factors for a given compound measured at two different temperatures (T_1 and T_2), respectively, and k'_3 is the capacity factor of this compound at the temperature of interest (T_3) in the interval of temperatures defined by T_1 and T_2. This equation permits the prediction of capacity factors by carrying out only two measurements, with an error not greater than 3%, and therefore within the experimental errors characteristic of liquid chromatography. Given that this equation does not require the calculation of specific coefficients dependent on the nature of the compound or the column, and given that Dufek affirms its general applicability (i.e.,

is not restricted to homologous series) it was of interest to verify to what extent this equation holds true for the 22 considered species in the two columns used and with the gradients described in Table 1.

For this purpose, a computer program was developed to check the extent of agreement of the available data with Dufek's equation for all the possible combinations of temperature values in the considered range (14—29°C). For each peak, the program selects two values of temperature, and with the k' data for these temperatures, the Dufek equation was applied to obtain the k' values for the rest of the temperature data. The results of these calculations in each case were compared with the experimental values of k' for the corresponding temperature, thus establishing the absolute differences between the calculated and experimental data and also the relative error at each temperature of the value given by Dufek's equation, according to the expression $100\ (k'\ (\text{exp.}) - k'\ (\text{calc.}))/k'\ (\text{exp.})$. A new pair of temperatures is chosen and the process is continued in the same manner until all possible combinations of pairs of temperature values have been used.

The results of these calculations were expressed in a histogram, representing the percentage of trials which led to a specific relative error (between 0.1 and 12%) compared with the experimental data. These histograms are reproduced in Fig. 2 for the two columns considered. With the μBondapak column, a very high degree of agreement with the equation is observed. Approximately 90% of the combinations tried gave errors less than or equal to 3%, and in no case were errors above 12% found. The maximum in the error distribution is at 3%, precisely the value given by Dufek [7]. Analogous results, although exhibiting somewhat lesser agreement, were obtained with the Novapak column, so that it can be affirmed that Dufek's equation can be used to predict the effect of temperature on gradient elutions, or can be introduced in simulation models for the optimization of HPLC gradient separations, no matter what the complexity of the separations or the particle size of the chromatographic column.

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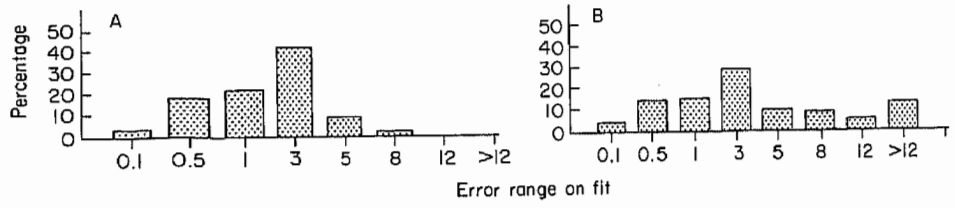

Fig. 2. Histograms of the relative error between the experimental data and those obtained through Dufek's equation: (A) μBondapak column; (B) Novapak column.

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