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EFFECT OF COLUMN TEMPERATURE ON HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC BEHAVIOUR OF INORGANIC POLYPHOSPHATES

I. ISOCRATIC ION-EXCHANGE CHROMATOGRAPHY

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SUMMARY

The isocratic elution conditions, column temperature and eluent concentration, at pH 10 for the analysis of inorganic polyphosphates were optimized to minimize analysis time and to maximize resolution. The retention time was described as a function of eluent concentration. The column temperature affected drastically both the retention time and the band broadening of polyphosphates. The enthalpy change associated with the retention was calculated to be positive. A computer-assisted retention prediction system based on these relationships was successfully applied to the optimization of the elution conditions. The graphical presentation of the optimal elution conditions provided a simple technique for obtaining an adequate set data on the column temperature and eluent concentration for the complete separation and rapid analysis of inorganic polyphosphates.

INTRODUCTION

Much interest has been focused on the reaction of inorganic polyphosphates in solution because of their important role in biosynthesis and metabolism[1–4], and their utility in various fields of industry and medicine[3–5]. High-performance liquid chromatography (HPLC) has been recognized as a powerful tool for the analysis of inorganic polyphosphates in solution[6–9] and has been applied to the kinetic research[10].

In the practical applications of HPLC two important problems are how to maximize sample resolution and how to minimize analysis time[11]. Many workers[12–15] have reported the effect of the concentration and the pH of the eluent on the chromatographic behaviour of inorganic polyphosphates. On the other hand, little attention has been paid to the column temperature, which may play a key role on their chromatographic behaviour[16].

The general relationship between retention time and eluent concentration in isocratic elution has been briefly described, and the drastic effect of the column temperature on retention time and band broadening has been shown in more detail. Furthermore, the combined effect of column temperature and eluent concentration has been discussed with the aid of a computer-assisted retention prediction system[17]. It was concluded that the column temperature, as well as the eluent concentration, plays an important role in the optimization of elution conditions.

EXPERIMENTAL

Chemicals and reagents

Unless otherwise stated, guaranteed reagents from Katayama (Osaka, Japan) and Kishida (Osaka, Japan) were used without further purification. Sodium phosphate glass (a mixture of $Na_{n+2}P_nO_{3n+1}$; $P_{\bar{n}}$) with an average polymerization number ($\bar{n}$) of 6 was prepared by thermal dehydration of orthophosphate.

A molybdenum(V)–molybdenum(VI) reagent for HPLC experiments was prepared by the method described previously[10] with slight modification. About 5.3 g of ammonium molybdate, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, were dissolved in *ca.* 700 ml of distilled water. After the dissolution of molybdate, 100 ml of concentrated sulphuric acid were slowly added. Metallic zinc (sandy, 0.65 g) was then added to the acidic Mo(VI) solution. Some of the Mo(VI) was reduced to Mo(V) after complete dissolution of zinc. The solution was diluted to 1 l with distilled water to give an orange Mo(V)–Mo(VI) reagent solution.

The eluents for the chromatographic separation of polyphosphates were composed of appropriate concentrations of potassium chloride and 0.1% (w/v) Na_4EDTA (pH 10).

Apparatus

A diagram of an HPLC flow injection analysis (FIA) system is shown in Fig. 1. A Hitachi Liquid Chromatograph 635 was used. The sample solution (100 μl) was introduced via a loop-valve sampler (S; Hitachi 635-5101) onto a separation column

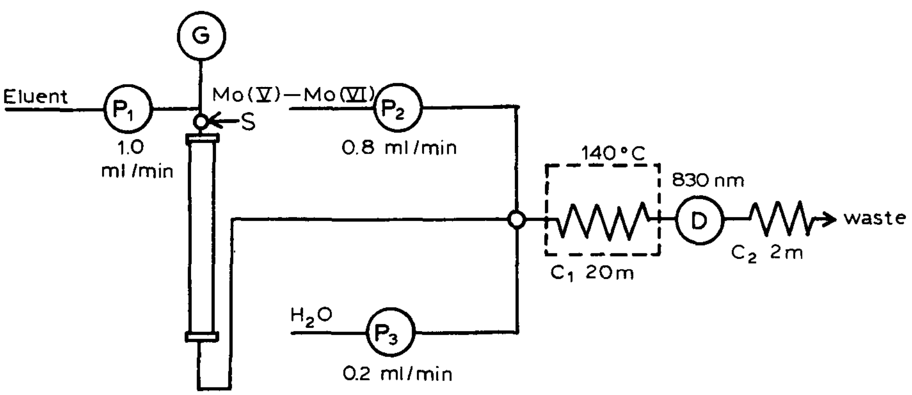

Fig. 1. HPLC–FIA system. P_1 = HPLC system (Hitachi 635); G = pressure gauge; S = sample injector; P_2 and P_3 = reciprocating pumps; C_1 = reaction coil (PTFE, 0.5 mm I.D.); D = detector, C_2 = back-pressure coil (PTFE, 0.25 mm I.D.). The part of the manifold enclosed by the dashed line was immersed in a thermostatted silicone oil-bath kept at 140°C.

and chromatographed at a flow-rate of 1.0 ml/min. The separations were performed on a column (250 × 4.0 mm I.D.) packed with a polystyrene-based quaternary ammonium anion-exchanger (TSKgel SAX, $d_p = 10$ μm, Toyo Soda).

The temperature of the separation column was controlled by a water-jacket of constant temperature (± 0.1°C, refrigerated circulating bath RTE-8, Neslab, U.S.A.). The column pressure drop increased from 20 to 50 kg/cm^2 as the column temperature fell from 80 to 5°C.

The effluent was introduced into an FIA system to permit the hydrolysis of polyphosphates to orthophosphate and the subsequent colour development of orthophosphate. The absorbance of the heteropoly blue complex at 830 nm was monitored by use of a spectrophotometric detector (D) with a flow-through cell. A channel (P_3) was reserved for multi-purpose use and was not always necessary in this case. Details of the HPLC–FIA system were described previously[18,19].

Measurement of capacity factor

Prior to the elution of polyphosphates the column was washed with the eluent for 1 h. The column dead time, t_0, was measured by injecting water. When an FIA system was used as a post-column reaction detector, extracolumn effects took place to some extent in HPLC[6,7,18]. Therefore the capacity factor, k', of a solute was calculated from its retention time[11], allowing for the extracolumn effects.

Calculation

All predicted retention times and band widths were calculated with a personal computer PC-9801 (NEC, Tokyo). The program for this purpose was written in BASIC and details of the program are described elsewhere[17]. The full listings may be available on the request to the authors.

RESULTS AND DISCUSSION

Capacity factor as a function of eluent concentration

The eluent concentration is one of the most important parameters controlling retention in ion-exchange chromatography. Many workers[13,14,20,21] have reported that the relationship between capacity factor, k', and the eluent concentration, C, can be expressed as follows,

$$k' = aC^{-b} \tag{1}$$

where a and b are constants. Fig. 2 shows chromatograms for the mixture of polyphosphates with eluent concentrations ranging from 0.23 to 0.34 M potassium chloride at constant column temperature (25°C). The retention time for each solute decreased with an increase in eluent concentration. The lower eluent concentrations were found to permit the good resolution of all solutes.

The plots of log k' *vs.* log C gave straight lines with correlation coefficients between 0.985 and 0.999 (Fig. 3). The constants b and a listed in Table I were calculated from the slopes and the intercepts at $C = 1$, respectively. The slopes were found to increase with increasing polymerization number of the polyphosphates. On the other hand, the slopes were found to be unaffected by the column temperature.

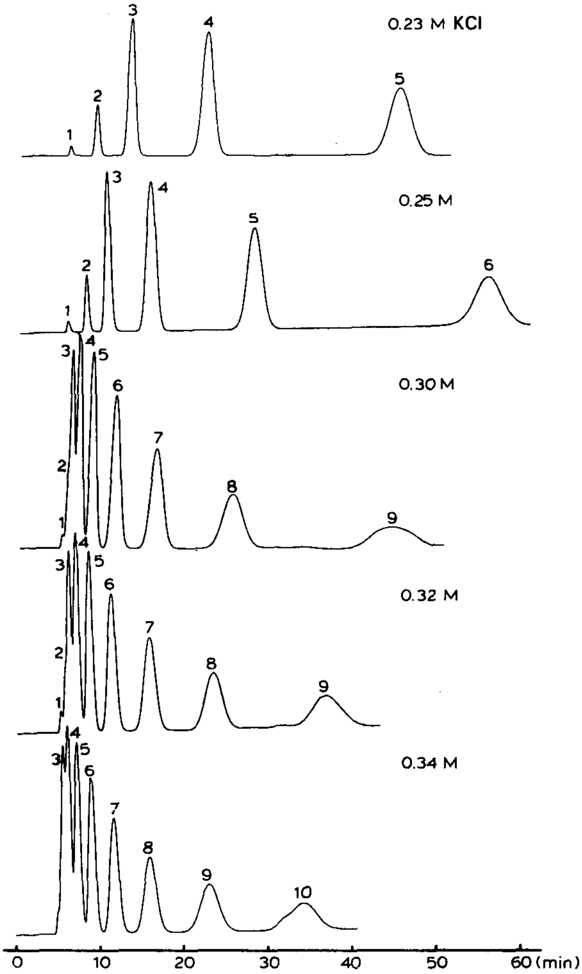

Fig. 2. Effect of changing eluent concentration on the elution profiles of inorganic polyphosphates at pH 10 and a column temperature of 25°C. Peaks numbers indicate polymerization numbers: 1 = ortho-; 2 = di-; 3 = tri-; 4 = tetra-; 5 = penta-; 6 = hexa-; 7 = hepta-; 8 = octa-; 9 = nona-; 10 = decaphosphate. Column, 250 × 4.0 mm I.D., porous strong anion exchanger (TSKgel SAX, 10 μm, 3.7 meq/g). Flow-rate, 1.0 ml/min.

The slope, b, is known to correlate with the charge of the solute in the ion-exchanger phase[21]. The charge of each solute with a polymerization number of n, except for orthophosphate, was expected to be $n+2$ at pH 10 because each solute with a first protonation constant of less than 10^9 was unprotonated[22,23]. The b values in Table I were in good agreement with the theory. The charge of orthophosphate was calculated from the first and the second protonation constants ($K_1 = 10^{10.8}$ and $K_2 = 10^{6.5}$ at 25°C) to be 2.1. This value agreed with the observed slope (25°C).

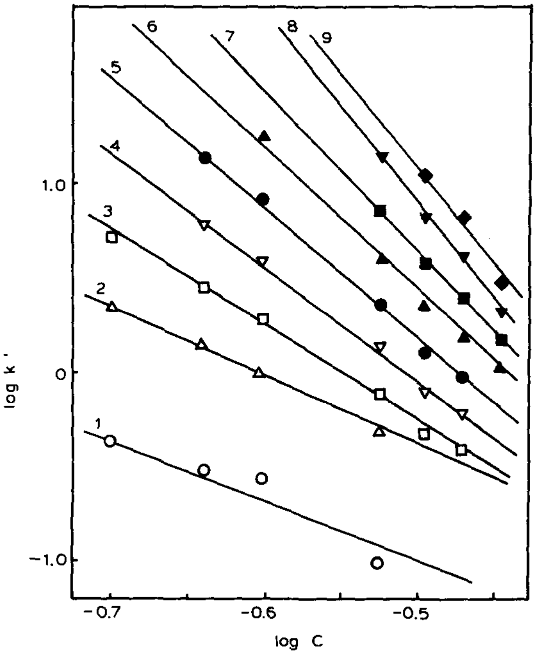

Fig. 3. Plots of log k' *vs.* log C at 25°C. Numbering as in Fig. 2.

Column temperature effect on retention and resolution of inorganic polyphosphates

Fig. 4 shows chromatograms for the mixture of polyphosphates obtained at various column temperatures from 20 to 60°C at constant eluent concentration (0.30 *M* potassium chloride). A marked temperature effect was observed on the chro-

TABLE I

VALUES OF CONSTANTS *a* AND b IN EQN. 1 AT VARIOUS COLUMN TEMPERATURES

*Solute**	*Column temperature (°C)*					
	25		*60*		*70*	
	b	*a* · 10^4	*b*	*a* · 10^4	*b*	*a* · 10^4
1	2.10	147	3.68	29.6	3.70	37.5
2	3.83	51.1	4.71	31.8	4.62	43.4
3	5.01	18.0	5.38	26.4	5.38	34.1
4	6.01	9.40	6.18	19.9	6.15	27.1
5	6.97	5.30	7.17	12.7	7.18	17.3
6	7.77	3.72	8.32	7.01	8.41	8.93
7	8.54	2.52				
8	10.2	0.662				
9	11.3	0.316				

* The numerals indicate the polymerization number of inorganic polyphosphates as shown in Fig. 2.

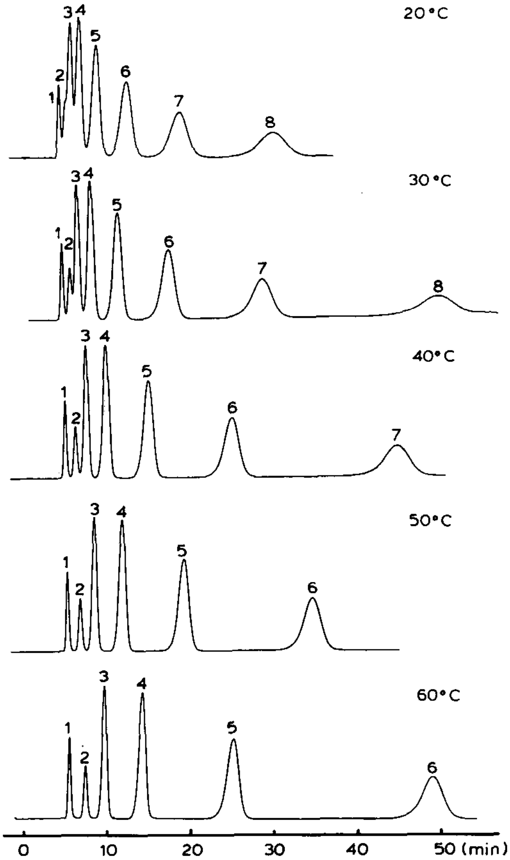

Fig. 4. Effect of column temperature on the elution profile of inorganic polyphosphates at pH 10. Eluent, 0.30 *M* potassium chloride and 0.1% Na_4EDTA. Column, flow-rate and numbering as in Fig. 2.

matographic behavior of each solute. The retention time increased as the column temperature increased. The retention times of the higher polyphosphates were more strongly affected than those of the lower. Elution at higher temperatures led to good resolution of all the solutes.

The column temperature is related to the capacity factor in the ion-exchange process as follows:

$$\ln k' = -\Delta H/RT + \Delta S/R + \beta \tag{2}$$

where ΔH and ΔS are the enthalpy and entropy changes, respectively, associated with the solute retention process. The parameter β is assumed to be constant for a given ion exchanger at a given eluent concentration.

The van 't Hoff plots for seven phosphates gave straight lines (Fig. 5), each with a correlation coefficient of 0.999. Enthalpy changes for polyphosphates were evaluated to be positive (Table II). The result is contrary to the temperature effect

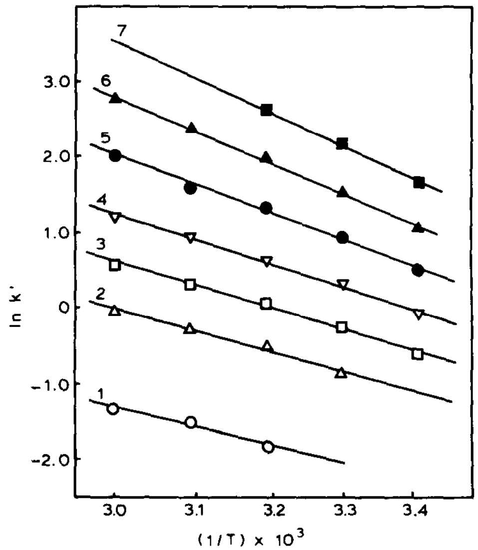

Fig. 5. Plots of ln k' *vs.* $(1/T) \times 10^3$. Numbering as in Fig. 2, eluent as in Fig. 4.

on the ion-exchange chromatographic behaviour of organic compounds[16,24], but is consistent with that of inorganic ions[25]. The positive ΔH values for polyphosphates may be ascribed to the less hydrophobic interaction of polyphosphate anions with the ion exchanger, compared with that of organic compounds.

The resolution R_s is expressed in terms of retention time and band width as follows:

$$R_s = 2(t_2 - t_1)/(w_1 + w_2) = (t_2 - t_1)/2(\sigma_1 + \sigma_2) \quad (3)$$

TABLE II

VALUES OF ΔH AT VARIOUS ELUENT CONCENTRATIONS

*Solute**	*Eluent concentration (M)*		
	0.20	*0.25*	*0.30*
	ΔH (kJ mol^{-1})	*ΔH (kJ mol^{-1})*	*ΔH (kJ mol^{-1})*
1	13.8	17.5	22.3
2	15.7	18.3	22.0
3	20.3	21.7	24.2
4	24.7	25.2	26.8
5		29.3	30.4
6		34.4	34.2
7			38.0

* The numerals indicate the polymerization number of inorganic polyphosphates as shown in Fig. 2.

where t_1 and t_2 are the observed retention times of bands 1 and 2, and w_1 and w_2 are their baseline band widths (in time units). Their standard deviations, σ_1 and σ_2, are approximately equal to one fourth of the w_1 and w_2 values, respectively. When the value of R_s is larger than 1.25, the separation is almost complete[11].

To evaluate the effect of elution conditions on resolution we must know how R_s varies with the capacity factor and the plate number. The fundamental equation, derived by Snyder and Kirkland[11], is:

$$R_s = (1/4)(\alpha - 1)N^{1/2}[k'/(1 + k')] \tag{4}$$

where N is the plate number, $\alpha(= k'_2/k'_1)$ is the separation factor and k'_1 and k'_2 are the capacity factors for bands 1 and 2. The quantity k' is the average value of k'_1 and k'_2. The three factors, α, N and k' control the resolution. The effect of the temperature on k' was described in the previous section.

It is evident from Fig. 4 that the band broadening of any peak appearing at a fixed retention time decreases as the temperature increases; the N value increases with increasing temperature[26,27]. The plate number[11] was found to be proportional to the column temperature: $N = 23.3\ T - 6500$ (T has the dimension of absolute temperature) with a correlation coefficient of 0.993 (Fig. 6). The separation factor, α, was also found to increase with increase in temperature (Fig. 4). For example, the separation factor between di- and triphosphate increased from 1.59 at 20°C to 1.88 at 60°C. Thus an increase in the column temperature advantageously affected both factors, α and N, so that the resolution was greatly improved.

The resolution between di- and triphosphate is always poorer than between any other pair of solutes under all elution conditions at pH 10 (Figs. 2 and 4). When the R_s value between di- and triphosphate was larger than 1.25, the resolutions of all

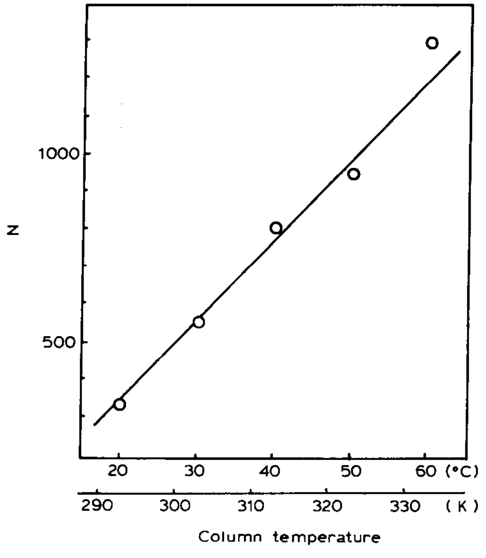

Fig. 6. Effect of column temperature on plate number N for the analysis of inorganic polyphosphates.

other pairs were achieved completely. Therefore only the lowest R_s value between di- and triphosphate is henceforth taken into account as a criterion for good resolution.

Inorganic polyphosphates are relatively labile substances in aqueous solutions. Griffith and Buxton[28] reported the rate constants for hydrolysis of polyphosphates containing from four to eight phosphorus atoms per molecule. They pointed out that the rate increased in proportion to the number of phosphorus atoms per molecule. For example, half-lives of tetra- and octaphosphate were determined to be 7900 and 630 min (60°C, pH 11), respectively. In time-consuming conventional chromatography[12,13], there is the possibility that unstable polyphosphates will decompose on the column. In HPLC, however, oligophosphates could be analysed in an hour without significant decomposition (less than 1%), even at 80°C.

As shown in Figs. 2 and 4 there is a practical limit, in that the peaks of the higher polyphosphates become too broad to be quantitated[29] when the capacity factor is more than *ca.* 20 for isocratic elution on a column with an N value of 1000. Therefore we recommend gradient elution for the analysis of higher polyphosphates[30].

Optimization of elution conditions using a computer-assisted retention prediction system

The elution conditions, column temperature and eluent concentration, were optimized with the aid of computer-assisted retention prediction system[17]. Details of the procedures for the optimization will be discussed elsewhere[31].

Fig. 7 illustrates the combined effect of column temperature and eluent con-

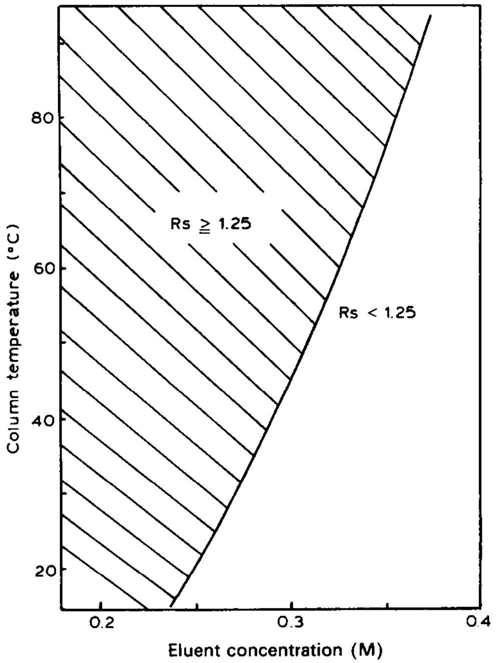

Fig. 7. Optimal elution conditions as a function of column temperature and eluent concentration at pH 10. R_s is the resolution between di- and triphosphate.

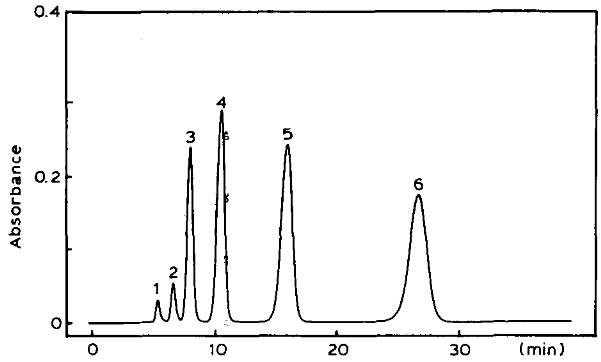

Fig. 8. Elution profile under the optimal conditions. Eluent, 0.35 *M* potassium chloride and 0.1% Na_4EDTA (pH 10). Column temperature, 80°C. Column, flow-rate and numbering as in Fig. 2.

centration of the resolution of di- and triphosphate at pH 10. Complete resolution of all solutes was expected under the elution conditions shown in shaded area, because the resolution between di- and triphosphate was the poorest of all as described in the previous section. The elution conditions necessary to minimize the analysis time without any reduction in resolution were further limited to the region close to the boundary of the shaded area because the analysis time increased as the eluent concentration decreased.

Fig. 8 shows an example of an elution profile under optimal elution conditions (80°C and 0.35 *M*). The six solutes could all be separated and analysed in less than 30 min. More complicated mixtures should be analysed under gradient elution conditions.

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