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## HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF LINEAR AND CYCLIC CONDENSED PHOSPHATES

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### SUMMARY

The separation of linear and cyclic condensed phosphates with a high-performance liquid chromatograph combined with an automatic phosphate analyser is described. Some experimental conditions were varied in order to obtain a good and reproducible separation of a series of condensed phosphates. More than 30 kinds of condensed phosphates have been separated by use of Hitachi 2630 ion-exchange resin (4% cross-linking), sodium chloride solutions containing 5 mM Na<sub>4</sub> EDTA as eluent and a gradient elution technique with a convex-type programme mode.

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### INTRODUCTION

Anion-exchange chromatography has been employed successfully for the separation of linear and cyclic condensed phosphates from one another<sup>1,2</sup>. However, these methods are laborious and time consuming because the effluent has to be divided into a large number of fractions and then the phosphorus content is determined manually in each effluent fraction. These drawbacks can be minimized by use of an automatic system for the detection of phosphates in the effluent from a chromatographic column, as described by Lundgren and Loeb<sup>3</sup> and others<sup>4-9</sup>.

On the basis of the above investigations, we have examined the possibilities and limitations of the separation of linear and cyclic condensed phosphates with different degrees of polymerization by combining a high-performance liquid chromatograph with an automatic phosphate detection system. It was important for the improvement of the resolution and the separation time to select properly the composition of the eluent and the degree of cross-linking of the anion-exchange resin.

It was found that the use of sodium chloride solutions containing di-, tri- or tetrasodium ethylenediaminetetraacetate (Na<sub>2</sub>, Na<sub>3</sub> or Na<sub>4</sub>) as the eluent resulted in an excellent separation of linear and cyclic condensed phosphates, whereas an ill-resolved and irreproducible chromatogram was obtained by use of sodium chloride solutions without the EDTA anion as the eluent<sup>7,9-12</sup>. A convex concentration gradient of sodium chloride in the eluent was more favourable than a linear gradient

with respect to the separation of linear condensed phosphates with a degree of polymerization of 10–30. An anion-exchange resin with low degree of cross-linking (4% divinylbenzene–polystyrene) was effective for the separation of condensed phosphates with a wide range of degree of polymerization from the monomer (orthophosphate) to about the 30-mer by use of an eluent containing 5 mM Na<sub>4</sub> EDTA, whereas that with a high degree of cross-linking (8% divinylbenzene–polystyrene) gave a good separation of condensed phosphates with a degree of polymerization of less than 10 or 11 with the same eluent.

## EXPERIMENTAL

### *Chemicals*

Reagent-grade trisodium orthophosphate dodecahydrate (Na<sub>3</sub>PO<sub>4</sub>·12H<sub>2</sub>O; P<sub>1</sub>) and tetrasodium diphosphate decahydrate (Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>·10H<sub>2</sub>O; P<sub>2</sub>) were used without purification. Pentasodium triphosphate hexahydrate (Na<sub>5</sub>P<sub>3</sub>O<sub>10</sub>·6H<sub>2</sub>O; P<sub>3</sub>) was purified by repeated recrystallization. Sodium phosphate glasses (a mixture of Na<sub>n+2</sub>P<sub>n</sub>O<sub>3n+1</sub>; P<sub>n</sub>) with average degrees of polymerization ( $\bar{n}$ ) of 5 and 10, trisodium trimetaphosphate trihydrate (Na<sub>3</sub>P<sub>3</sub>O<sub>9</sub>·3H<sub>2</sub>O; P<sub>3m</sub>), tetrasodium tetrametaphosphate tetrahydrate (Na<sub>4</sub>P<sub>4</sub>O<sub>12</sub>·4H<sub>2</sub>O; P<sub>4m</sub>) and hexasodium hexametaphosphate hexahydrate (Na<sub>6</sub>P<sub>6</sub>O<sub>18</sub>·6H<sub>2</sub>O; P<sub>6m</sub>) were prepared in our laboratory. These materials were dissolved in distilled water. All other chemicals were of reagent grade.

### *Apparatus and procedures*

A Hitachi Model 635 liquid chromatograph with a solvent programmer was used for the separation of linear and cyclic condensed phosphates. A water-jacketed glass column of 9 mm I.D. was packed with Hitachi 2630 or 2632 Custom ion-exchange resin to a height of 10 cm by the slurry-packing method. Nine kinds of stock solutions of sodium chloride with or without EDTA were prepared by dissolving the calculated amount of sodium chloride and Na<sub>4</sub>, Na<sub>3</sub> or Na<sub>2</sub> EDTA in 1 l of distilled water. Six types of eluents with different kinds of EDTA sodium salts and different concentration gradients of sodium chloride were fed to the chromatographic column by use of a solvent programmer with a flow-rate of 1 ml/min. The conditions are summarized in Table I.

After conditioning the resin column with eluent containing the initial concentration of sodium chloride for 30 min, 0.5 ml of a sample solution was added to the resin column with the aid of a loop injection valve and the chromatographic run was started. The changes in the sodium chloride concentration in the eluent for the different programme modes are shown in Fig. 1. The concentration of sodium chloride reached its final value after operation for 6 h in all of these programme modes, and then 1 M sodium chloride solution not containing EDTA was added to the resin column at a flow-rate of 1 ml/min. The column temperature was maintained at 31 ± 1°.

The effluent from the liquid chromatograph was introduced into a Technicon AutoAnalyzer II automatic phosphate detection system. This system is composed of five functional units, *viz.*, a proportionating pump, a heating bath for the hydrolysis of condensed phosphates, a heating bath for the colour development of molybdo-phosphate heteropoly blue, a colorimeter and a recorder, as shown in Fig. 2. The

TABLE I

## COMPOSITIONS OF ELUENTS AND PROGRAMME MODES FOR GRADIENT ELUTION

Eluent No.	NaCl concentration of the solution in reservoir (M)		EDTA sodium salt and pH value in reservoirs A and B		Programme mode	NaCl concentration in the eluent (M)	
	A	B	Na <sub>x</sub> EDTA	pH		Initial	Final
1	0.25	0.53	Without EDTA	5.5	Convex <sup>*</sup>	0.25	0.53
2	0.22	0.60	5 mM Na <sub>4</sub> EDTA	10.0	Linear <sup>**</sup>	0.22	0.56
3	0.22	0.60	5 mM Na <sub>4</sub> EDTA	10.0	2-step linear <sup>***</sup>	0.22	0.49
4	0.22	0.53	5 mM Na <sub>4</sub> EDTA	10.0	Convex <sup>§</sup>	0.22	0.53
5	0.22	0.53	5 mM Na <sub>3</sub> EDTA	7.5	Convex <sup>§</sup>	0.22	0.53
6	0.22	0.53	5 mM Na <sub>2</sub> EDTA	4.4	Convex <sup>§</sup>	0.22	0.53

<sup>\*</sup> C-1 in Fig. 1.

<sup>\*\*</sup> L-1 in Fig. 1.

<sup>\*\*\*</sup> L-2 in Fig. 1.

<sup>§</sup> C-2 in Fig. 1.

reagents used were 3.45 M sulphuric acid,  $8.1 \cdot 10^{-3}$  M ammonium molybdate solution containing 0.6 M sulphuric acid, 0.1 M L-ascorbic acid containing 50 ml of acetone and 5 ml of 10% Aerosol-22 solution per litre of solution and surfactant solution containing 5 ml of 10% Aerosol-22 solution per litre of solution. These solutions and air were passed into the AutoAnalyzer II, together with a stream of the chromatographic effluent with the set of flow-rates (case 1) as designated in Fig. 2 and its modification (case 2) with respect to the flow-rates of effluent intake and surfactant solution, respectively. It was confirmed that a phosphate species was detected on the recorder 18 min after entry into the AutoAnalyzer II.

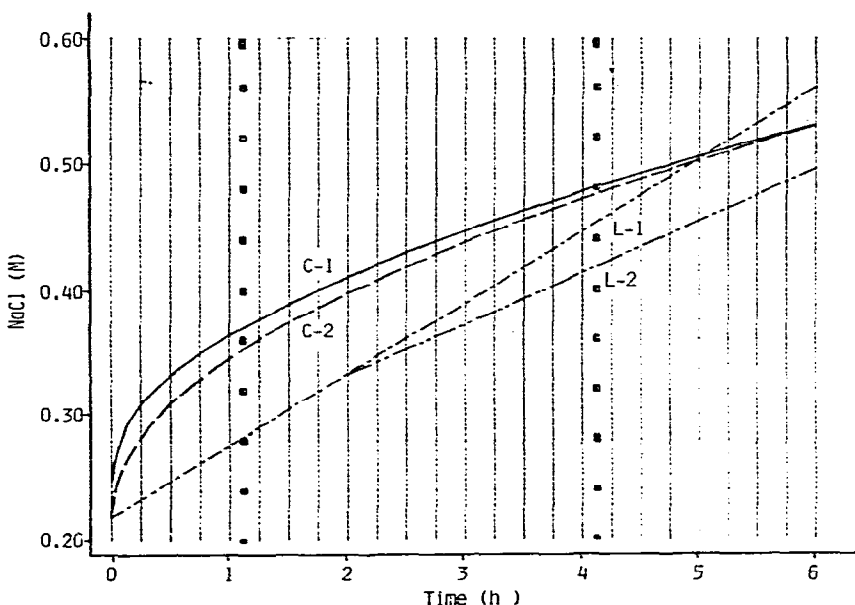


Fig. 1. Changes in sodium chloride concentration in the eluents for different programme modes (L-1, L-2, C-1 and C-2, where L = linear and C = convex).

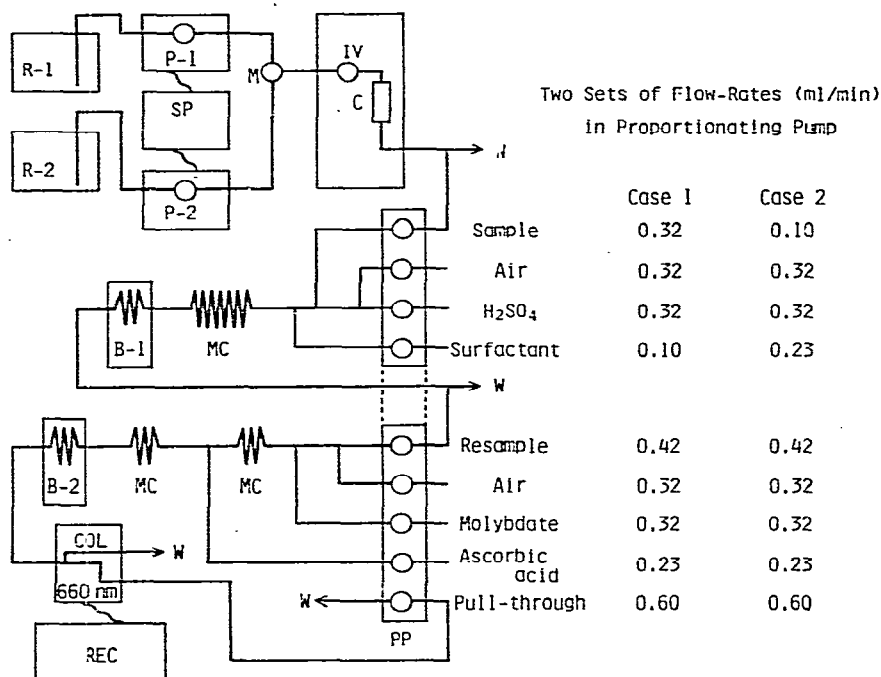


Fig. 2. Scheme of the separation-detection system (Hitachi Model 635 liquid chromatograph-Technicon AutoAnalyzer II). R-1 and R-2, reservoirs; SP, solvent programmer; P-1 and P-2, dual-piston high-pressure pumps; M, mixer; IV, injection valve; C, glass column with anion-exchange resin, W, waste; PP, proportionating pump; MC, mixing coil; B-1, heating bath for hydrolysis of condensed phosphates (97°); B-2, heating bath for colour development (80°); COL, colorimeter; REC, recorder. Two sets of flow-rates (cases 1 and 2) are given for sample, air and reagents.

In order to examine the chromatographic behaviour of the EDTA anion when using 0.22–0.53 *M* sodium chloride solution (5 *mM* Na<sub>4</sub> EDTA) with the convex mode, the remaining effluent after intake into the AutoAnalyzer II was introduced into an automatic fraction collector with a time collection device, which was set to move at 5-min intervals. Each effluent fraction in a test-tube was diluted with 5 ml of distilled water and the absorbance due to the EDTA anion was measured at 250 nm with a Hitachi Model 100-20 spectrophotometer.

## RESULTS AND DISCUSSION

### *Effect of the addition of EDTA sodium salts to the eluent*

Although the addition of EDTA sodium salts to an eluent has been applied in the conventional anion-exchange chromatographic separation of phosphorus oxo-anions<sup>11,12</sup>, Nakae *et al.*<sup>8</sup> have also reported that the addition of Na<sub>2</sub> EDTA to the eluent was effective in the high-performance liquid chromatographic separation of ortho-, di- and triphosphates in detergents in reducing the tailing of the peaks for di- and triphosphates. On the basis of these results, we examined the chromatographic behaviour of condensed phosphates by use of eluents with and without EDTA. Fig. 3 is a typical anion-exchange chromatogram obtained by the present system

with an eluent not containing EDTA (eluent 1). The chromatograms obtained in two other runs under the same conditions were different from each other and from that shown in Fig. 3.

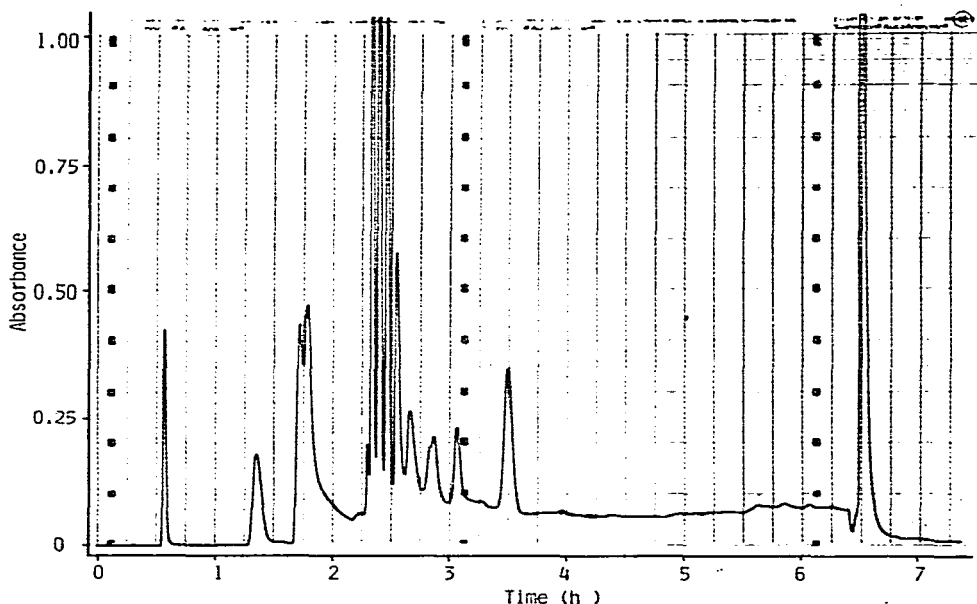


Fig. 3. Chromatogram of  $P_1$ ,  $P_2$ ,  $P_n(\bar{n} = 5)$  and  $P_n(\bar{n} = 10)$  with eluent 1 and Hitachi 2630 anion-exchange resin.

On the other hand, the use of an eluent containing 5 mM  $Na_4$  EDTA gave a good and reproducible separation of a series of condensed phosphates. Three eluents of this type (eluents 2, 3 and 4) were used, and the chromatograms were compared. Eluent 4 gave the best chromatogram with respect to the separation of a series of linear condensed phosphates from ortho- to polyphosphate with a degree of polymerization of *ca.* 30, as shown in Fig. 4.

The order of elution of three kinds of cyclic condensed phosphates (trimeta-, tetrameta- and hexametaphosphates) was determined by separate chromatographic runs under the same conditions as those in Fig. 4. The results are shown in Fig. 5. Each of these cyclic condensed phosphates was eluted considerably later than the linear condensed phosphates with the same number of phosphorus atoms, *i.e.*, tri-, tetra- and hexaphosphate, and the order of elution was the reverse of the order of increasing anionic charge. However, these results are in accordance with those obtained by Grande and Beukenkamp<sup>13</sup> and by Tominaga *et al.*<sup>14</sup> in anion-exchange chromatography with potassium or sodium chloride solution as the eluent and that obtained by Kura and Ohashi<sup>15</sup> in anion-exchange chromatography with QAE-Sephadex A-25 gel using potassium chloride solution as the eluent.

From a comparison of the chromatograms in Figs. 3 and 4, it is clear that the addition of  $Na_4$  EDTA to the eluent is necessary for the separation of condensed phosphates as described in our previous work<sup>11,12</sup>. Here, the chromatographic behaviour of the EDTA anion is of interest for its competitive function with chloride

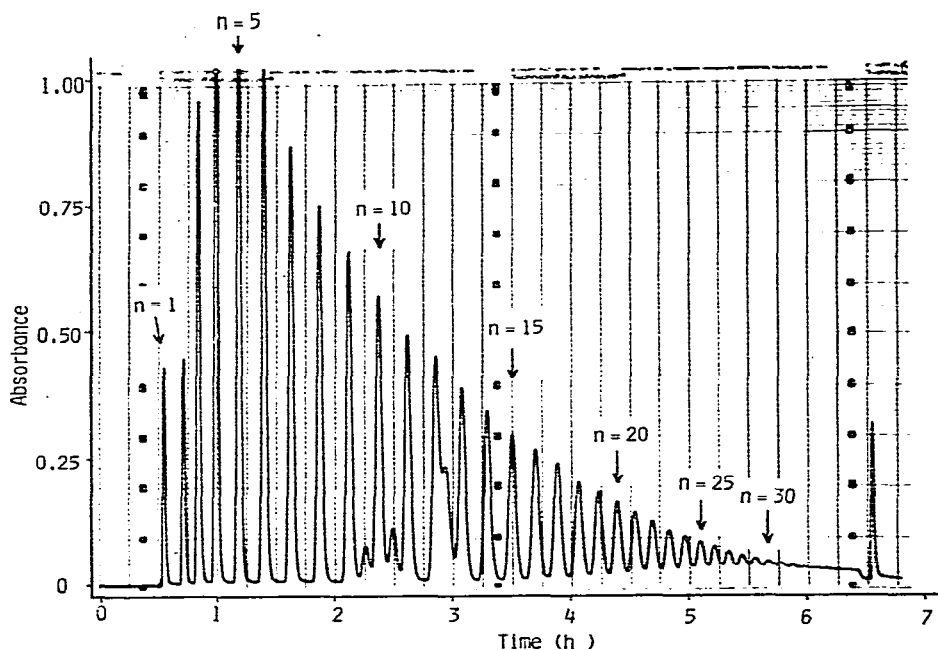


Fig. 4. Chromatogram of  $P_1$ ,  $P_2$ ,  $P_n(\bar{n} = 5)$  and  $P_n(\bar{n} = 10)$  with eluent 4 and Hitachi 2630 anion-exchange resin.

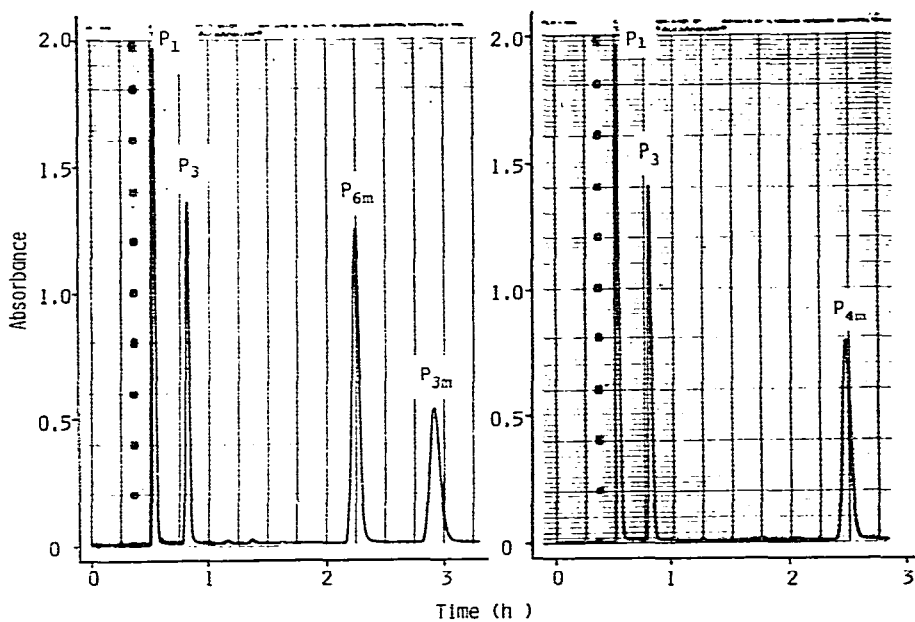


Fig. 5. Chromatograms of  $P_1$ ,  $P_3$ ,  $P_{3m}$  and  $P_{6m}$  (left), and of  $P_1$ ,  $P_3$  and  $P_{4m}$  (right), with eluent 4 and Hitachi 2630 anion-exchange resin.

ion as the eluting agent for phosphate anions. The change in the concentration of EDTA anion in the effluent is shown in Fig. 6. The results indicate that EDTA anions in the eluent occupy preferentially the cationic sites in the anion-exchange resin at sodium chloride concentrations in the eluent lower than about 0.3 M, but are removed from the resin by eluents with higher sodium chloride concentrations, and then the concentration of EDTA anions in the effluent reaches a constant value expected to be the same as that in the eluent.

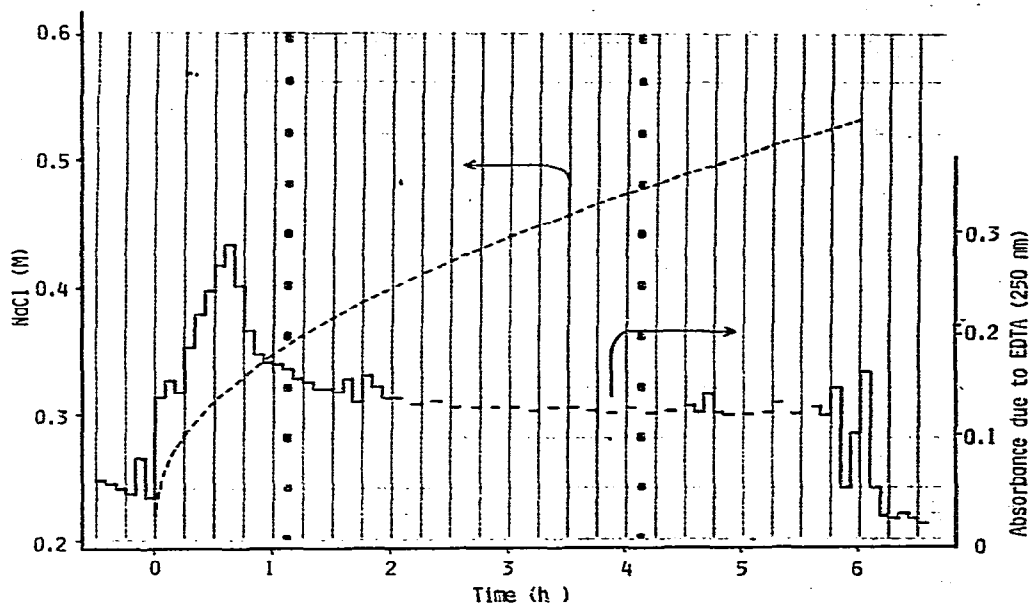


Fig. 6. Chromatographic behaviour of EDTA anion with eluent 4 and Hitachi 2630 anion-exchange resin.

#### *Effect of the degree of cross-linking of the anion-exchange resin on the separation of condensed phosphates*

It is said that ion-exchange resins with lower degrees of cross-linking show higher efficiencies but lower selectivities than those with higher degrees of cross-linking and swell or shrink with changes in the experimental conditions such as mobile phase ionic strength, although they are suitable for the ion-exchange chromatographic separation of large molecules such as polypeptides and nucleotides<sup>16</sup>.

We have applied a Hitachi 2632 anion-exchange resin with 8% cross-linking for the separation of condensed phosphates because of the lower degree of swelling and shrinking with changes in the sodium chloride concentration in the eluent and the successful application of Durrum DA-X8 resin in the separation of linear condensed phosphates from ortho- to octaphosphate by Deelder *et al.*<sup>9</sup>. Fig. 7 is a chromatogram of a series of condensed phosphates obtained by use of a Hitachi 2632 anion-exchange resin and eluent 2 with a linear programme mode. Comparison of this chromatogram with that in Fig. 4 indicates that the separation of condensed phosphates with a degree of polymerization greater than 12 is unsuccessful, whereas a good separation is obtained for less polymerized chain phosphates from ortho- to undecaphosphate. These results suggest that the pore sizes in this anion-exchange

resin are not large enough for large phosphate anions to pass through them freely. These steric effects can be confirmed by the fact that the use of an anion-exchange resin with lower degree of cross-linking (Hitachi 2630) resulted in a good separation of condensed phosphates from orthophosphate to large polyphosphates with a degree of polymerization of *ca.* 30, as shown in Fig. 4. Although Hitachi 2630 anion-exchange resin is more difficult to handle than Hitachi 2632 resin because of the higher degree of swelling and shrinking of the former, a resin column packed by the slurry-packing method and conditioned with 1.0 *M* sodium chloride solution gives good and reproducible chromatograms of condensed phosphates.

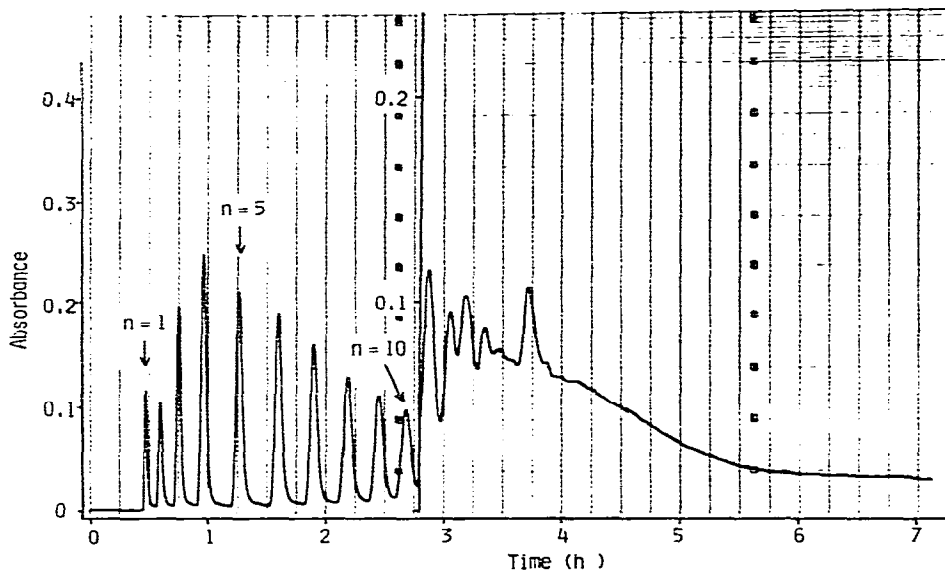


Fig. 7. Chromatogram of  $P_1$ ,  $P_2$ ,  $P_n(\bar{n} = 5)$  and  $P_n(\bar{n} = 10)$  with eluent 2 and Hitachi 2632 anion-exchange resin.

#### *Effect of different EDTA sodium salts in the eluent on the chromatographic behaviour of condensed phosphates*

As mentioned above, the inclusion of 5 mM  $Na_4$  EDTA in the eluent gave a good and reproducible separation of condensed phosphates. We also examined the chromatographic behaviour of condensed phosphates on a Hitachi 2630 anion-exchange resin column with an eluent containing 5 mM  $Na_3$  or  $Na_2$  EDTA. Figs. 8 and 9 are the chromatograms obtained with eluents containing 5 mM  $Na_3$  EDTA (eluent 5) and 5 mM  $Na_2$  EDTA (eluent 6), respectively. When using eluent 6, the elution was continued for 1 h by holding the mixing ratio of the solutions in reservoirs A and B at that at the end of the 6-h operation, *i.e.*, 0.53 *M* sodium chloride with 5 mM  $Na_2$  EDTA. The elution positions of trimeta-, tetrameta- and hexametaphosphates were also determined in the separate chromatographic runs and are designated in Figs. 8 and 9. From the chromatograms in Figs. 4, 8 and 9, it can be seen that the elution positions of linear condensed phosphates shift to later values with a decrease in the pH of the eluent, whereas those of cyclic condensed phosphates remain approximately constant. These results are in accordance with those expected ones from the



pH dependence of the distribution ratios obtained by Beukenkamp *et al.*<sup>17</sup>, although they investigated the chromatographic behaviour of only ortho-, di-, tri-, trimeta- and tetrametaphosphates at different pH values and chloride ion concentrations.

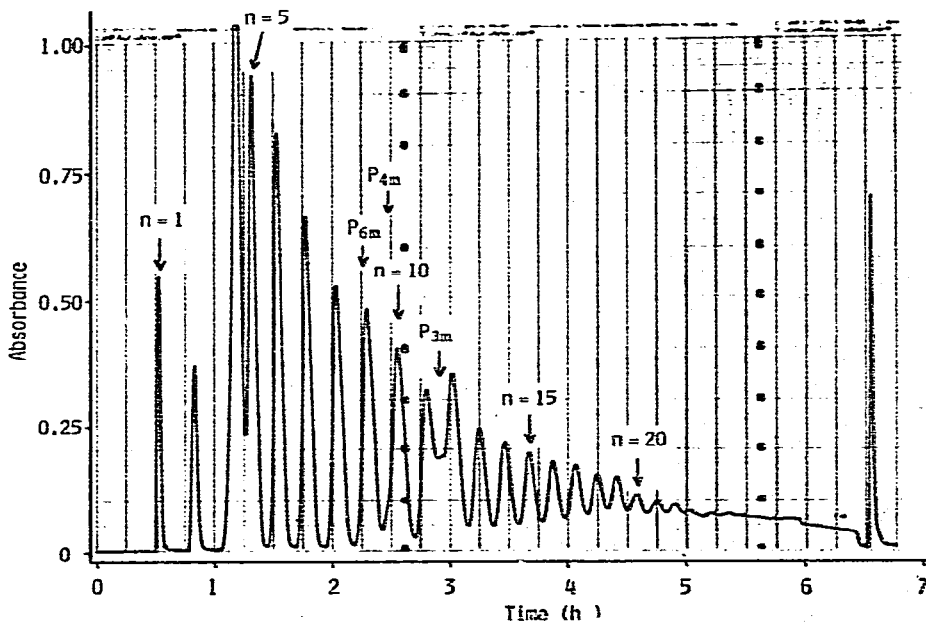


Fig. 8. Chromatogram of  $P_1$ ,  $P_2$ ,  $P_n(\bar{n} = 5)$  and  $P_n(\bar{n} = 10)$  with eluent 5 and Hitachi 2630 anion-exchange resin.

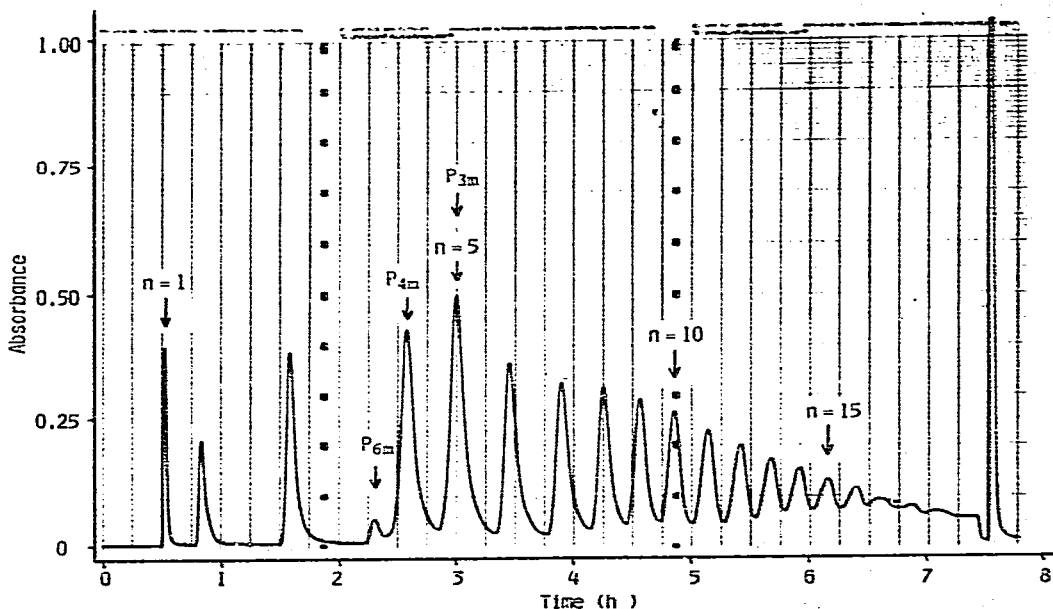


Fig. 9. Chromatogram of  $P_1$ ,  $P_2$ ,  $P_n(\bar{n} = 5)$  and  $P_n(\bar{n} = 10)$  with eluent 6 and Hitachi 2630 anion-exchange resin.

From the view point of a practical separation technique for condensed phosphates, the eluent containing 5 mM Na<sub>2</sub>EDTA is most useful of the three kinds of eluents examined, and the other two eluents give complementary information.

#### ACKNOWLEDGEMENTS

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