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OPTIMIZATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF INORGANIC POLYPHOSPHATES FOR ROUTINE ANALYSES

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SUMMARY

The practical advantages of using an isocratic elution technique in the routine analysis of orthophosphate, diphosphate, triphosphate and tetraphosphate by high-performance liquid chromatography with an anion-exchange column and a post-column detector were studied. A parameter "sample width" is introduced in expressing the efficiency of a chromatographic system in isocratic elution. The routine analysis of six samples per hour was possible for orthophosphate, diphosphate and triphosphate. Two examples of applications are presented: a purity check on tetraphosphate and preparative and kinetic investigations of phosphorus compounds.

INTRODUCTION

In previous papers[1,2] we reported two types of post-column detector for the high-performance liquid chromatography (HPLC) of inorganic polyphosphates. Two methods were described, based on the formation of the so-called heteropoly blue complex by reaction of orthophosphate with molybdenum reagents but differing in the construction of the analytical manifolds, with an air-segmented flow system[1] and an unsegmented flow system[2]. Both methods were confirmed to be satisfactory with respect to sensitivity and reproducibility when each detector was connected with an HPLC column packed with an anion exchanger. The practical utility of the AutoAnalyzer II (Technicon) air-segmented system for routine analyses is considered in this paper.

If a sample to be analysed contains a complex mixture of polyphosphates, it is usually recommended that a technique of gradient elution by which more than 20 components can be separated is employed[3,4]. On the other hand, an isocratic elution technique becomes advantageous if the sample composition is not complex. For example, a mixture of orthophosphate (P_1), diphosphate (pyrophosphate, P_2) and triphosphate (tripolyphosphate, P_3) can easily be separated[1,2]. A great advantage of isocratic elution over gradient elution is that samples can be injected successively without reconditioning the column for each run, with a consequent reduction in the

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time of analysis. This advantage may be very significant for analysts who are engaged in the routine analyses of many samples from industrial, biological, clinical, agricultural and environmental sources[5]. The rapid determination of orthophosphate, diphosphate and triphosphate is also frequently required in many academic and industrial laboratories for characterizing the reaction products in preparative and kinetic experiments.

Elution profiles at various eluent concentrations and at pHs 8 and 10 are presented in this paper for the convenience of practical users who want to carry out the routine analysis of orthophosphate, diphosphate, triphosphate and tetraphosphate (tetrapolyphosphate, P_4) by using an anion-exchange column packed with TSK-GEL, IEX-220SA.

EXPERIMENTAL

Samples

Disodium hydrogen orthophosphate ($Na_2HPO_4 \cdot 12H_2O$) and tetrasodium diphosphate ($Na_4P_2O_7 \cdot 10H_2O$) were guaranteed reagents from Wako (Osaka, Japan). Pentasodium triphosphate ($Na_5P_3O_{10} \cdot 6H_2O$) from Wako was purified by repeated recrystallization. Hexammonium tetraphosphate [$(NH_4)_6P_4O_{13} \cdot 6H_2O$] and disodium diphosphonate ($Na_2P_2H_2O_5$) were prepared according to the literature[6,7].

Eluents

Eluents of pH 10 consisted of $2 \cdot 10^{-3}$ *M* Na_4EDTA and appropriate concentrations of potassium chloride. Eluents of pH 8 consisted of $1 \cdot 10^{-3}$ *M* Na_4EDTA, $1 \cdot 10^{-3}$ *M* Na_2EDTA and appropriate concentrations of potassium chloride.

Apparatus and procedures

A Hitachi Model 635 liquid chromatograph was used. Sample solution (100 μl) was injected into the separation column (500 × 2.6 mm I.D.) packed with TSK-GEL, IEX-220SA anion exchanger and chromatographed at a flow-rate of 1.0 ml/min and a pressure of about 150 kg/cm^2. A portion of the effluent (0.6 ml/min) was introduced into the AutoAnalyzer II as described in previous papers[1,2] 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 measured using a spectrophotometric detector with a flow-through cell (light path = 15 mm).

RESULTS AND DISCUSSION

Elution at pH 10

Retention times of polyphosphates are well known to be dependent not only on the properties of the column packings, but also on the concentration of chloride ions in eluents. Therefore, it is necessary to examine the variation of elution profiles with chloride concentration, using a chromatographic system of interest. Elution profiles for a mixture of orthophosphate, diphosphate, triphosphate and tetraphosphate are shown in Figs. 1–4 which were obtained by isocratic elution at pH 10 on an anion-exchange column packed with TSK-GEL, IEX-220SA. Na_4EDTA was

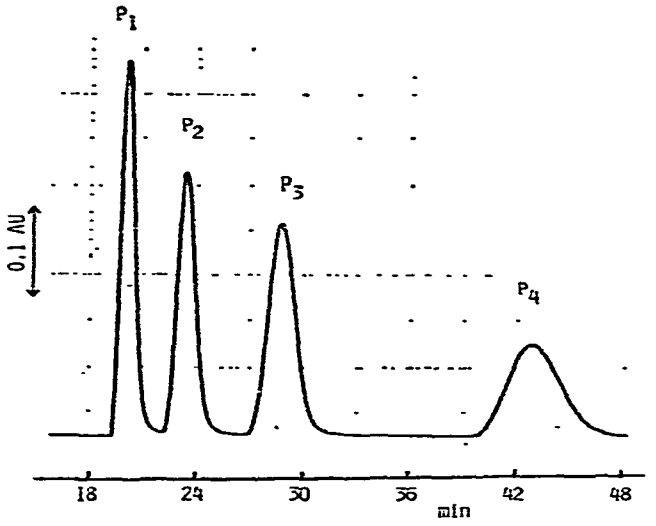


Fig. 1. Elution profile for a mixed solution of orthophosphate (P_1), diphosphate (P_2), triphosphate (P_3) and tetraphosphate (P_4). Eluent, 0.21 *M* KCl (pH 10); sample size, about 40 nmol each (as P); detection, 1 a.u.f.s.

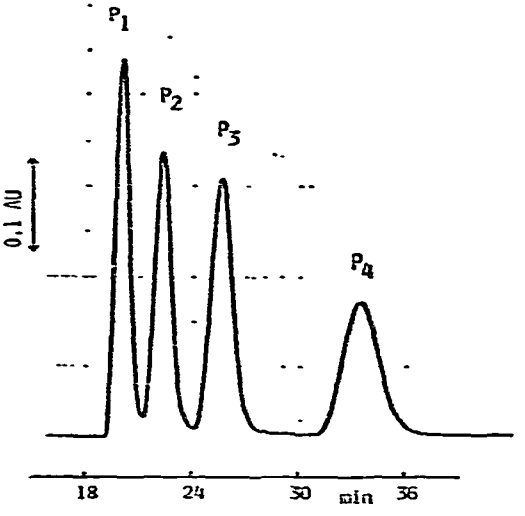
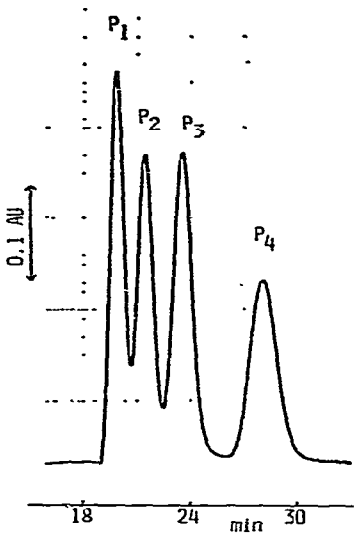


Fig. 2. Elution profile for a mixed solution of orthophosphate (P_1), diphosphate (P_2), triphosphate (P_3) and tetraphosphate (P_4). Eluent, 0.23 *M* KCl (pH 10); other conditions as in Fig. 1.

added to the eluent to adjust the pH (to about 10.5) and to mask the interfering metal ions that may be present in the eluent. As shown in Figs. 1–4, the retention time for each component, except orthophosphate, decreased with increase in chloride concentration from 0.21 to 0.27 *M*. The eluents with lower chloride concentrations (Figs. 1 and 2) were found to permit the good resolution of four components. At 0.3 *M* potassium chloride all components overlapped to form a broad peak with a shoulder corresponding to tetraphosphate.



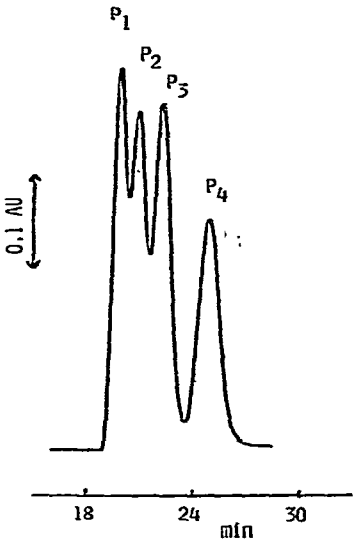


Fig. 3. Elution profile for a mixed solution of orthophosphate (P_1), diphosphate (P_2), triphosphate (P_3) and tetraphosphate (P_4). Eluent, 0.25 *M* KCl (pH 10); other conditions as in Fig. 1.

Fig. 4. Elution profile for a mixed solution of orthophosphate (P_1), diphospate (P_2), triphosphate (P_3) and tetraphosphate (P_4). Eluent, 0.27 *M* KCl (pH 10); other conditions as in Fig. 1.

The retention time for orthophosphate (about 20 min) remained almost constant as the chloride concentration varied. This value was considered to correspond to the retention time of a non-retained component or the void retention time. The residence time of orthophosphate in the separation system was calculated to be about 2 min, because the residence time of the sample in the manifold of the AutoAnalyzer II was about 18 min, which was necessary to achieve the hydrolysis of polyphosphates to orthophosphates and the colour reaction of the resultant orthophosphate with a molybdenum reagent[1,2]. The residence time in the AutoAnalyzer II was not always reproducible from day to day; with fluctuations of $\pm$ 0.5 min. Therefore, it is recommended that the void retention time be checked before and after a series of experimental runs by elution of orthophosphate as a standard, if the retention times of polyphosphates are to be evaluated exactly.

If there is only one sample to be analysed, it is a great disadvantage that the void retention time is as long as 18 min. This problem becomes less serious, however, when many samples with the same components are submitted to routine analysis, because with isocratic elution the samples can be injected successively at appropriate time intervals that are long enough to avoid carry over between adjacent samples.The time intervals for successive injections should not be shorter than the "sample width", $W_{F,L}$, defined by

$$W_{F,L} = t_L - t_F + \tfrac{1}{2}(w_F + w_L) \qquad (1)$$

where t and w represent the retention time and the peak width (4σ), respectively, of the first peak (F) and the last peak (L) in a sample of interest. For example, $W_{F,L}$ for

orthophosphate, diphosphate, triphosphate and tetraphosphate in Fig. 2 is calculated to be about 17 min. Most biological, agricultural and industrial samples to be analysed include only orthophosphate, diphosphate and triphosphate, except tetraphosphate[7]. Fig. 2 suggests that the routine analysis of six samples per hour is possible in such instances, as the sample width for P_1, P_2 and P_4 in 9 min. If only tetraphosphate is to be evaluated the elution system in Fig. 4 becomes convenient to permit rapid analyses with $W_{F,L} = 7$ min.

Elution at pH 8

Polyphosphates composed of only phosphorus (V), such as diphosphate, triphosphate and tetraphosphate described above, are relatively stable to hydrolysis during chromatographic elution at pH 10. As there are some polymeric oxo acids of phosphorus containing phosphorus (III) and phosphorus (IV) that are less stable in alkaline solution, it is necessary to develop chromatographic methods in neutral media. The pH of the eluent was adjusted to about 8 with an equimolar mixture of Na_4EDTA and Na_2EDTA. Elution profiles for a mixture of orthophosphate, diphosphate, triphosphate and tetraphosphate are shown in Figs. 5–8. At 0.23 *M* chloride concentration all components were well resolved, with a broad sample width of $W_{F,L} = 26$ min. Although the sample widths decreased with increase in chloride concentration, triphosphate and tetraphosphate tended to overlap at 0.25 *M*, with complete overlap at 0.27 and 0.30 *M*.

The elution system at pH 8 had the drawback that a very long time was required to achieve the equilibrium of the column with the eluent. For example, reproducible results were not obtained until about 100 ml of the eluent, corresponding

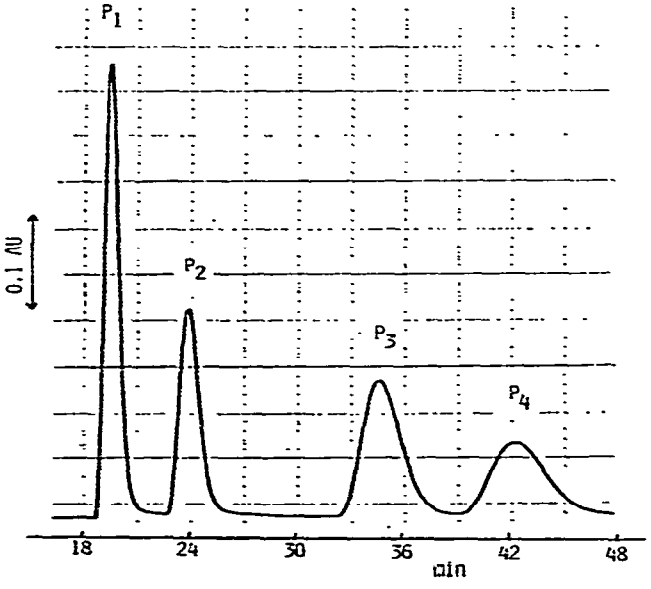


Fig. 5. Elution profile for a mixed solution of orthophosphate (P_1), diphosphate (P_2), triphosphate (P_3) and tetraphosphate (P_4). Eluent, 0.23 *M* KCl (pH 8); sample size, about 40 nmol each (as P); detection, 1 a.u.f.s.

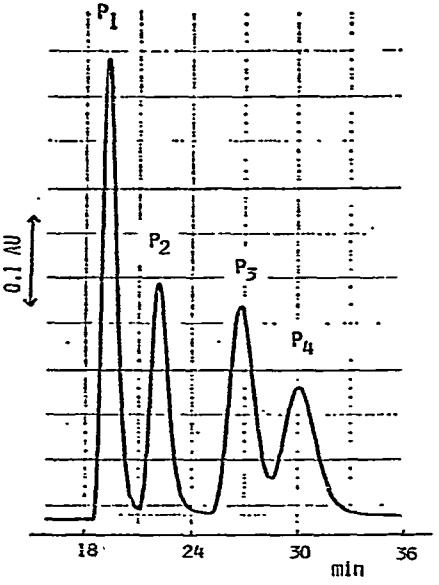


Fig. 6. Elution profile for a mixed solution of orthophosphate (P_1), diphosphate (P_2), triphosphate (P_3) and tetraphosphate (P_4). Eluent, 0.25 *M* KCl (pH 8); other conditions as in Fig. 5.

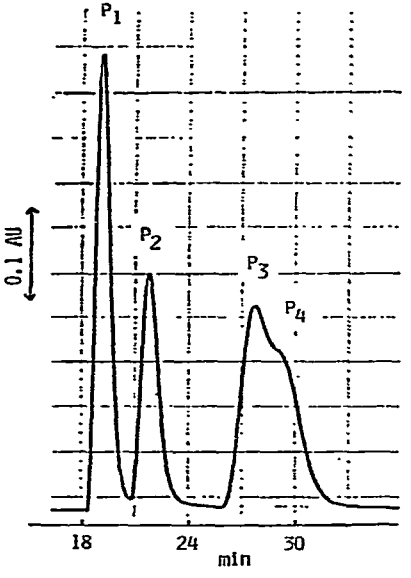


Fig. 7. Elution profile for a mixed solution of orthophosphate (P_1), diphosphate (P_2), triphosphate (P_3) and tetraphosphate (P_4). Eluent, 0.27 *M* KCl (pH 8); other conditions as in Fig. 5.

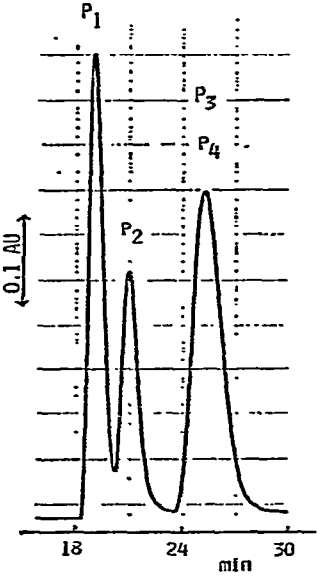


Fig. 8. Elution profile for a mixed solution of orthophosphate (P_1), diphosphate (P_2), triphosphate (P_3) and tetraphosphate (P_4). Eluent, 0.30 *M* KCl (pH 8); other conditions as in Fig. 5.

to 40 times the column volume, had passed through the separation system at a flow-rate of 1 ml/min. As the elution profiles varied gradually with the progress of conditioning, careful observation was needed in order to distinguish whether the column conditioning was complete or not by repeated injection of the same standard sample. If a sample is stable in alkaline solution, elution systems at pH 10 are recommended because the equilibrium for conditioning is attained within 15 min and a high resolution and good reproducibility can be obtained at pH 10.

Purity check on tetraphosphate

Tetraphosphate can be prepared by the thermal dehydration of orthophosphate[6] and the crude product is purified by repeated crystallization. During such purification a rapid and convenient means of monitoring the contaminants such as orthophosphate, diphosphate and triphosphate is necessary. Similar techniques are also frequently required in order to examine the stability of this compound during its storage, especially in an aqueous solution. Paper and thin-layer chromatographic techniques are simple, but are not satisfactory for quantitative purposes. Classical column chromatographic techniques with ion-exchange and gel chromatographic packings have been widely employed for the quantitative determination of phosphorus compounds[5,8]. However, in gel chromatography the resolution between triphosphate and tetraphosphate is poor[9] and in ion-exchange chromatography either gradient or stepwise elution must be employed to achieve the separation of orthophosphate, diphosphate, triphosphate and tetraphosphate. These disadvantages were overcome in this work by using TSK-GEL, IEX-220SA (Fig. 2). The porosity of this anion exchanger, with $-N(CH_3)_3^+$ groups, is assumed to be well controlled so as to give advantageous sieving characteristics. Combined contributions of both anion-exchange and steric mechanisms may be a factor leading to acceptable separations of orthophosphate, diphosphate, triphosphate and tetraphosphate by isocratic elution. Fig. 9 shows the elution profile for an aqueous solution of tetraphosphate (0.01 *M*) stored in a refrigerator for 1 week. By comparing it with the standard profile in Fig. 2 it was found that orthophosphate, diphosphate and triphosphate were produced as contaminants during the storage of tetraphosphate.

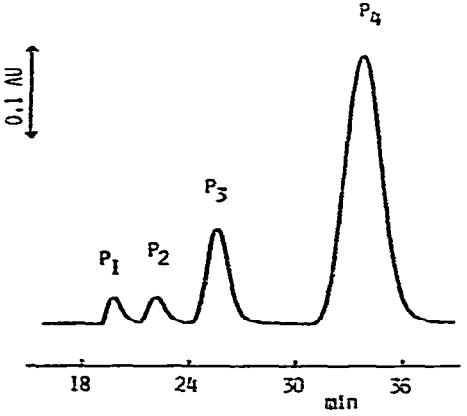
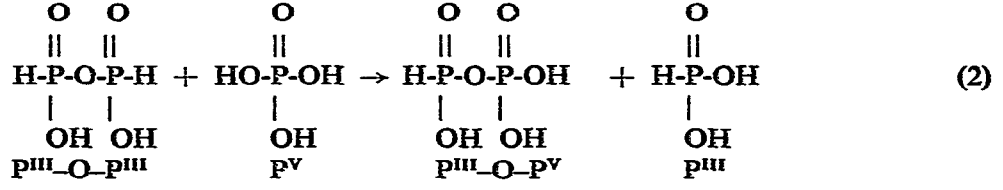


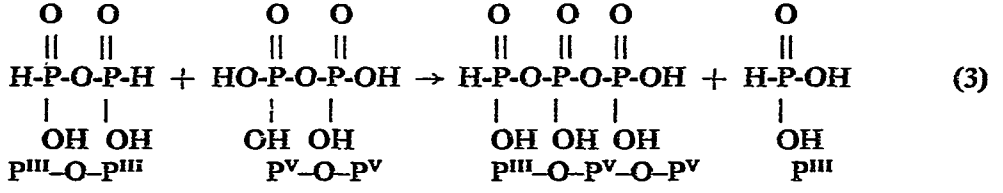
Fig. 9. Purity check on tetraphosphate (P_4). Eluent, 0.23 *M* KCl (pH 10); sample size, about 100 nmol (as P); detection, 1 a.u.f.s.

Preparative and kinetic experiments

Substitution reactions between oxo acids of phosphorus have been systematically investigated for the synthesis of novel compounds[7,10,11]. For example, orthophosphate (P^V) and diphosphate (P^V–O–P^V) react with diphosphonate (P^{III}–O–P^{III}), to form compounds with a terminal P^{III} unit according to the following reactions*:

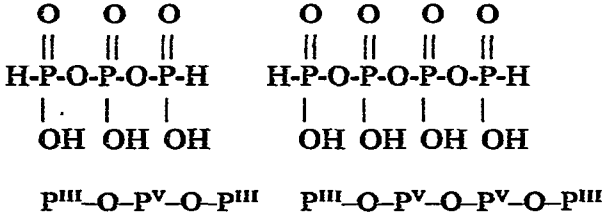


(2)



(3)

Further reactions of this type lead to the formation of the following compounds with two terminal P^{III} units:



The formation of these compounds has been characterized by gel chromatography, using a fraction colletor combined with a batchwise colorimetric method[6,10,11]. This time-consuming procedure was not suitable for the kinetic experiments, which required the rapid analysis of phosphorus compounds in the various steps of the reactions. The elution system in Fig. 5 was employed to permit successive injections of a series of analytes for kinetic experiments.

A mixed solution of P^{III}–O–P^{III} and P^V, each about 0.25 *M*, was allowed to stand at 50°C. At appropriate time intervals aliquots of this mixture were diluted and chromatographed in order to follow the reaction products according to eqn. 2. The detection method employed in this experiment was selective to P^V units, but was insensitive to P^{III} units. For example, P^V and P^{III}–O–P^V in eqn. 2 can be detected with identical molar absorptivities, but P^{III} and P^{III}–O–P^{III} do not respond. As expected, only a peak for orthophosphate was observed when a mixed solution

* To avoid confusion on nomenclature, Blaser and Worms' notation[12] is used for phosphorus compounds in this section. The superscripts III and V represent the oxidation state of phosphorus.

of orthophosphate and diphosphonate was analysed immediately after mixing (Fig. 10a). The peak of orthophosphate decreased gradually with time to give the peak of P^{III}–O–P^{V} (Fig. 10b and c).

Kinetic experiments were also carried out in the same way with a mixed solution of P^{III}–O–P^{III} and P^{V}–O–P^{V} according to eqn. 3. As shown in Fig. 11, the formation of P^{III}–O–P^{V}–O–P^{V} with a terminal P^{III} unit was clearly indicated on the chromatograms.

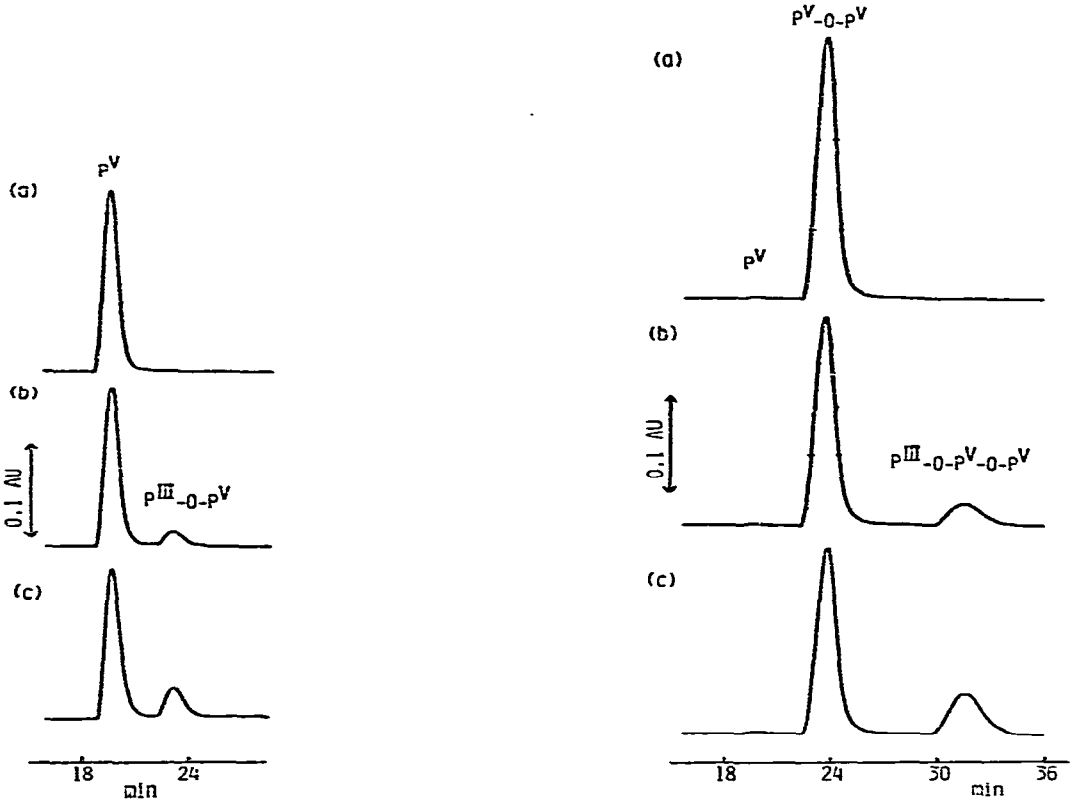


Fig. 10. Elution profiles for a mixed solution of P^{III}–O–P^{III} and P^{V}. Reaction times: (a) 0 min; (b) 120 min; (c) 300 min. Eluent, 0.23 *M* KCl (pH 8); sample size, about 25 nmol P^{V} units; detection, 1 a.u.f.s.

Fig. 11. Elution profiles for a mixed solution of P^{III}–O–P^{III} and P^{V}–O–P^{V}. Reaction times: (a) 0 min; (b) 60 min; (c) 180 min. Sample size, about 50 nmol P^{V} units; other conditions as in Fig. 10.

When the concentration of P^{III}–O–P^{III}, one of the starting materials, was increased from 0.25 to 1.25 *M*, without changing the concentrations of P^{V} and P^{V}–O–P^{V} described just above, P^{III}–O–P^{V}–O–P^{III} and P^{III}–O–P^{V}–O–P^{V}–O–P^{III} with two terminal P^{III} units appeared far behind the peaks of P^{III}–O–P^{V} and P^{III}–O–P^{V}–O–P^{V} in Figs. 10 and 11. The tetramer P^{III}–O–P^{V}–O–P^{V}–O–P^{III} had a retention time as high as 38 min even when the potassium chloride concentration in the eluent was increased to 0.3 *M*. Additional details of the chromatographic characteristics of these novel compounds will be presented elsewhere.

As mentioned above, the residence time of the sample in the AutoAnalyzer II is as long as about 18 min. In order to overcome this drawback another type of chemical detector based on an unsegmented flow system (flow injection system) has been developed in our laboratory[2]. Polyphosphates can be monitored quantitatively with residence times of less than 3 min in such a detector[13]. This type of detector is also promising for preparative and kinetic experiments.

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