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Rapid and sensitive determination of nucleoside H-phosphonates and inorganic H-phosphonates by high-performance liquid chromatography coupled with flow-injection analysis

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SUMMARY

A coupled high-performance liquid chromatographic (HPLC) and flow-injection analysis (FIA) system was developed for the separation and detection of H-phosphonates. The H-phosphonates are separated by HPLC in the anion-exchange mode and then introduced into an on-line FIA system. In the FIA system, H-phosphonate esters and anhydrides (P–O–P bonds) are hydrolysed and oxidized to orthophosphate, the resulting orthophosphate is converted into the heteropoly blue complex and the latter is monitored spectrophotometrically at 830 nm. The system is sensitive and easy to use. The detection limit is 10^{-6} M for inorganic, sugar and nucleoside H-phosphonates. The reproducibility of the peak areas was less than 1% (relative standard deviation) for all H-phosphonates.

INTRODUCTION

Nucleoside H-phosphonates are useful synthetic intermediates for the rapid synthesis of DNA and RNA[1–3]. H-phosphonates have also been widely applied in the synthesis of "biophosphates", such as phospholipid and nucleoside polyphosphates[4–9]. Few analytical methods, however, have been developed for the sensitive determination of H-phosphonates[10–15]. The development of an analytical method for

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H-phosphonates would open up new possibilities for characterizing the mechanisms of their synthetic reactions and designing methods for their preparation.

In this study, we developed a coupled high-performance liquid chromatographic (HPLC) and flow-injection analysis (FIA)[10-12] system, the latter being used for post-column reaction detection for the sensitive determination of H-phosphonate. Sodium hydrogensulphite[13-15] and an acidic solution containing molybdenum(V) and molybdenum(VI)[16,17] were used as an oxidizing agent and a chromogenic agent, respectively. In the post-column reaction detector, H-phosphonate is oxidized to orthophosphate and then the resulting orthophosphate is converted into a heteropoly blue complex which is detectable by spectrophotometry at 830 nm.

EXPERIMENTAL

Chemicals

Unless stated otherwise, guaranteed reagents from Wako (Osaka, Japan) and Yamasa Shoyu (Chiba, Japan) were used without further purification. The structures
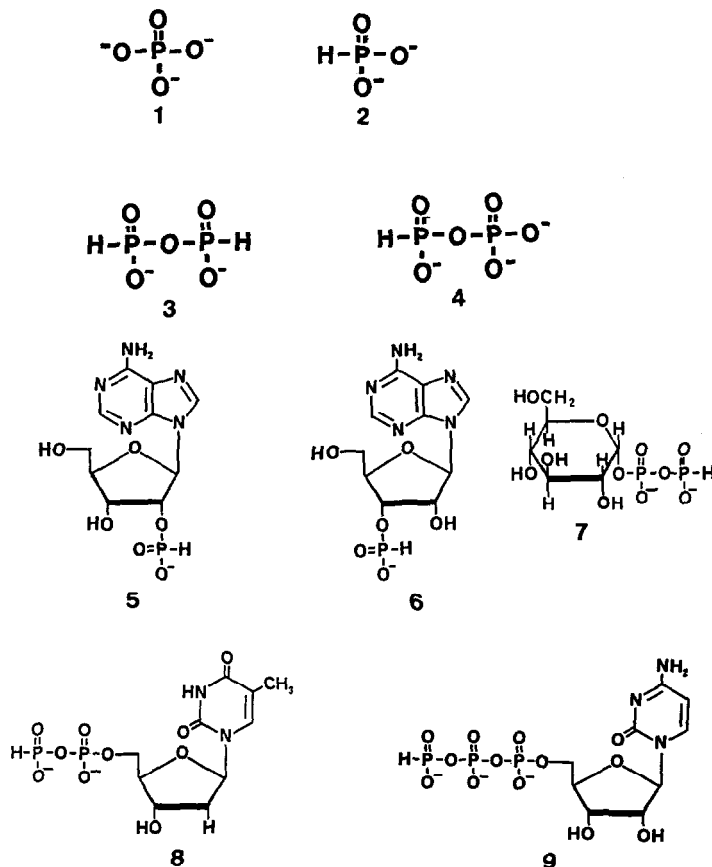

Fig. 1. Structures of orthophosphate (1), inorganic H-phosphonates (2–4), nucleoside H-phosphonates (5,6,8,9) and sugar H-phosphonate (7). Sodium salts of these compounds, which are illustrated as ionic forms, were used.

of H-phosphonate samples used are shown in Fig. 1. Disodium diphosphonate (**3**)[8], diphosphate(III, V) (**4**)[18], sugar H-phosphonate (**7**)[7] and nucleoside H-phosphonates (**5, 6, 8, 9**)[8,9] were prepared according to the literature. Water was purified with a Milli-Q system (Millipore, Bedford, MA, U.S.A.).

Reagents and eluents

Chromogenic reagent. An acidic solution containing molybdenum(V) and molybdenum(VI) was used as a chromogenic reagent for the determination of H-phosphonates. Such reagent is called a molybdenum(V)–molybdenum(VI) or Mo(V)–Mo(VI) reagent. The reagent was prepared as follows[16,17]. About 5.3 g of ammonium molybdate, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, were dissolved in *ca.* 700 ml of Milli-Q water. After the dissolution of molybdate [Mo(VI)], 100 ml of concentrated sulphuric acid (*ca.* 18 *M*) 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 Milli-Q water to give an orange Mo(V)–Mo(VI) reagent solution.

Oxidizing reagent. Sodium hydrogensulphite solution was used to oxidize the H-phosphonate group to phosphate, which is detectable by the Mo(V)–Mo(VI) reagent. A sulphite solution was prepared daily by dissolving 52 g of sodium hydrogensulphite in Milli-Q water and dilution to 500 ml.

Eluents. The eluents for the separation of H-phosphonates were composed of appropriate concentrations of potassium chloride and 0.1% (w/v) Na_4EDTA. The addition of EDTA is essential to prevent the distortion of chromatographic peaks, probably due to the hydrolysis of phosphoric anhydride bonds (P–O–P bonds) during the chromatographic process[19].

Equipment

The HPLC–FIA system is shown in Fig. 2. The HPLC system consisted of a Jasco 880-PU HPLC pump (P_A), an injection valve (S) (Sanuki SV1-5U7) and the separation column (250 mm × 4.0 mm I.D.) packed with an anion exchanger (TSKgel SAX, d_p = 10 μm; Tosoh). The FIA system consisted of a Sanuki FIA pump (2P2M-4027) with two channels (P_B and P_C), a six-way loop-valve injector (L) (Sanuki SVM-6M2), two detectors (D_A, Jasco UVIDEC-100-IV UV detector; D_B, Soma S-3250 spectrophotometric), two PTFE coils (RC, reaction coil, 20 m × 0.5 mm I.D.; BC, back-pressure coil, 1.5 m × 0.25 mm I.D.) and a thermostated reaction bath (Taiyo TAH-1).

Eluent (P_A), chromogenic reagent (P_B) and Milli-Q water (P_C) were pumped into the analytical lines at flow-rates of 1.0, 0.8 and 0.2 ml/min, respectively. The oxidizing reagent was introduced into the water stream via a six-way loop-valve injector (L), becuase the sulphite solution tended to corrode the stainless-steel pumping system. Sample solution (100 μl) was introduced with an injection valve (S) into the eluent stream. The separations were performed on the separation column. The effluent was introduced into the FIA system. Separated phosphonate species were mixed with chromogenic and oxidizing reagents, and then the UV absorption of nucleotide was monitored at 260 nm (D_A). Although the UV detector is located after the mixing point in Fig. 2, it can be located before the mixing point. The mixed solution was carried through a 20-m reaction coil maintained at 140°C, during which time (*ca.* 120 s)
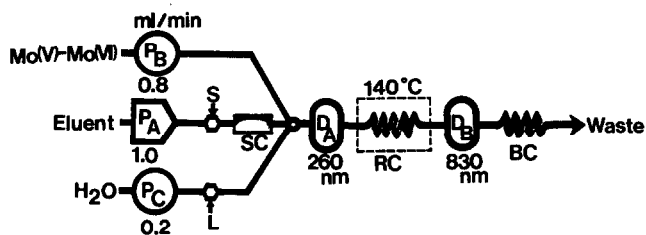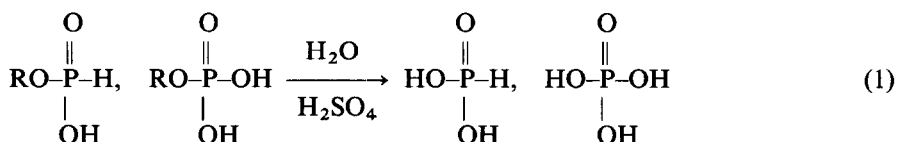
Fig. 2. HPLC–FIA system. P_A = HPLC pump (1.0 ml/min); P_B and P_C = FIA pumps with two pumping channels (0.8 and 0.2 ml/min); S = sample injector; L = sulphite injector; SC = separation column (TSK gel SAX anion exchanger, 250 mm × 4.0 mm I.D.); D_A = UV detector; RC = reaction coil (PTFE, 20 m × 0.5 mm I.D.); D_B = spectrophotometric detector; BC = back-pressure coil (PTFE, 1.5 m × 0.25 mm I.D.). The dashed line shows the reaction bath (Taiyo TAH-1), in which the reaction coil is tightly wound on a thermostated aluminium block kept at 140°C.

H-phosphonate esters and anhydrides were hydrolysed to orthophosphate and H-phosphonate, H-phosphonate was subsequently oxidized to orthophosphate and the resulting orthophosphate was allowed to react with the chromogenic reagent to form a heteropoly blue complex. The absorption of the blue complex at 830 nm was monitored by the spectrophotometer (D_B). The back-pressure coil (BC) at the exit of the spectrophotometer (D_B) prevented baseline noise due to gas bubbling even at temperatures as high as 140°C.
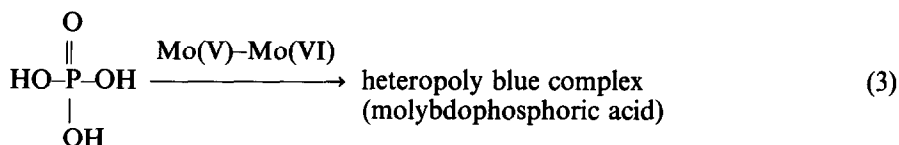
RESULTS AND DISCUSSION

FIA for post-column reaction detection

H-phosphonates injected into the HPLC–FIA system (Fig. 2) are separated by HPLC and detected in the on-line FIA system. The following three reactions occurred simultaneously in the FIA system: (1) hydrolysis of phosphoric (or phosphonic) ester and anhydride linkages catalysed by sulphuric acid (eqn. 1); (2) oxidation of H-phosphonate to orthophosphate with sulphite (eqn. 2); and (3) colour development of orthophosphate by Mo(V)–Mo(VI) reagent (eqn. 3). The oxidation of phosphonate with sulphite is well known[20-22], but its reaction mechanism is too complicated to be expressed by a simple equation.

$$\begin{array}{cc} \underset{\overset{|}{\text{OH}}}{\overset{\overset{\text{O}}{\parallel}}{\text{RO–P–H,}}} & \underset{\overset{|}{\text{OH}}}{\overset{\overset{\text{O}}{\parallel}}{\text{RO–P–OH}}} \end{array} \xrightarrow[\text{H}_2\text{SO}_4]{\text{H}_2\text{O}} \begin{array}{cc} \underset{\overset{|}{\text{OH}}}{\overset{\overset{\text{O}}{\parallel}}{\text{HO–P–H,}}} & \underset{\overset{|}{\text{OH}}}{\overset{\overset{\text{O}}{\parallel}}{\text{HO–P–OH}}} \end{array} \qquad (1)$$

R = alkyl or phosphoric group

$$\underset{\overset{|}{\text{OH}}}{\overset{\overset{\text{O}}{\parallel}}{\text{HO–P–H}}} \xrightarrow{\text{sulphite}} \underset{\overset{|}{\text{OH}}}{\overset{\overset{\text{O}}{\parallel}}{\text{HO–P–OH}}} \qquad (2)$$

$$\underset{\overset{\displaystyle |}{OH}}{\overset{\overset{\displaystyle O}{\overset{\displaystyle \|}{}}}{HO-P-OH}} \xrightarrow{\text{Mo(V)--Mo(VI)}} \text{heteropoly blue complex} \qquad (3)$$
$$\text{(molybdophosphoric acid)}$$

To examine the extent of oxidation of H-phosphonate, inorganic orthophosphate (1) or inorganic phosphonate (2), the sample was introduced into the system (Fig. 2) without the separation column. Fig. 3 indicates that oxidation of phosphonate is quantitative in the 120-s residence time in the reaction coil, *i.e.*, the peak height at each concentration of phosphonate with sulphite is the same as that of each equimolar amount of orthophosphate, whereas no peak of phosphonate was detected without sulphite.

Fig. 3 also demonstrates that the differentiation of phosphate and phosphonate was easily achieved by use of the system, because both phosphate and phosphonate were detected in the presence of sulphite, whereas only phosphate was detected in the absence of sulphite.

The calibration graph for phosphonate with sulphite showed good linearity (correlation coefficient = 0.999) and the relative standard deviation of measurement was less than 1%. The detection limit was found to be *ca.* 10^{-6} *M* for phosphonate.
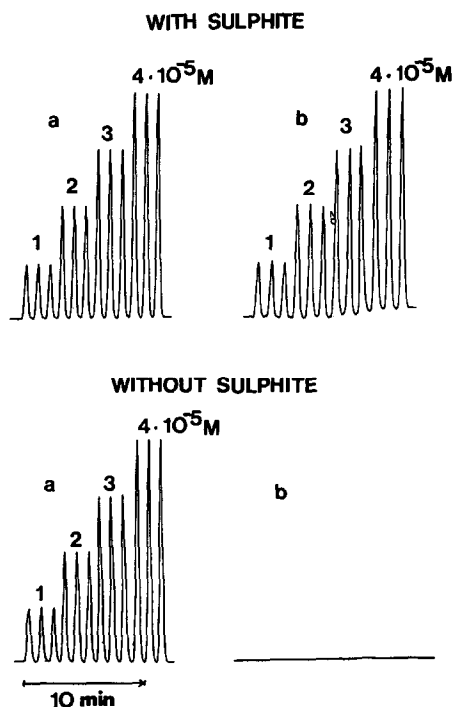

Fig. 3. FIA calibration profiles of (a) orthophosphate and (b) phosphonate with and without sulphite solution. The concentration of each sample increases from left to right, *i.e.*, from $1 \cdot 10^{-5}$ to $4 \cdot 10^{-5}$ *M*. Each sample was injected in triplicate. The anion-exchange column was not connected.

With orthophosphate detection, almost the same linearity, reproducibility and sensitivity were observed.

Analysis of inorganic H-phosphonates

Some inorganic phosphates and H-phosphonates were separated and detected using the HPLC–FIA system as shown in Figs. 4 and 5. Orthophosphate (**1**) and phosphonate (**2**) were almost separated (Fig. 4). The solid line in Fig. 4b represents the sum of the absorbances due to phosphate and phosphonate units measured in the presence of sulphite, and the dotted line represents the absorbance due to the phosphate unit measured in the absence of sulphite. The peak of orthophosphate was observed in both cases (solid and dotted lines) but that of posphonate only in the presence of sulphite (solid line). No peak was detected by the UV detector, as shown in Fig. 4a.

Fig. 5 shows the elution profile of the products and the reactants for the phosphonylation of orthophosphate (**1**) with inorganic diphosphonate (Fig. 7b). We recently found that diphosphonate (**3**). The reaction gave phosphonate (**2**) and diphosphate(III, V) (**4**) as products. Diphosphonate (**3**) was detected only with sulphite, because diphosphonate was the dimer of phosphonate as illustrated in Fig. 1. Diphosphate(III, V) (**4**), which is an anhydride of phosphate and phosphonate, is detected in both instances, as shown in Fig. 5b. The ratio of phosphate to phosphonate groups for **4** was calculated to be 1.0, which is expected from the structure in Fig. 1. Differentiation of phosphate and phosphonate by use of the HPLC–FIA system gave useful information for the establishment of the chemical structure.
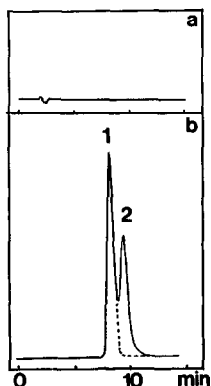

Fig. 4. Elution profiles of orthophosphate (**1**) and phosphonate (**2**). Eluent: 0.10 M KCl + 0.1% Na$_4$EDTA (pH 10). Wavelength: (a) 260 nm and (b) 830 nm. The solid line in (b) indicates total phosphate and phosphonate units with sulphite and the dotted line only the phosphate unit without sulphite. Peak numbers correspond to the compound numbers in Fig. 1.
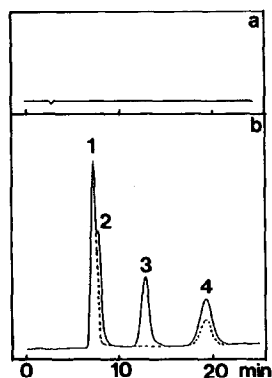
Fig. 5. Elution profiles for orthophosphate (**1**) phosphonylated by diphosphonate (**3**) at pH 6 and 60°C for 10 h. **2** = Phosphonate; **4** = diposphate(III,V). Eluent: 0.15 M KCl + 0.1% Na$_4$EDTA. Wavelength and meaning of the solid and dotted lines in (b) as in Fig. 4. Peak numbers correspond to the compound numbers in Fig. 1.

Analysis of sugar and nucleoside H-phosphonates

Figs. 6–9 illustrate the separation and detection of some nucleoside and sugar H-phosphonates. Such H-phosphonates (**5–9** in Fig. 1) are all prepared by the reaction of nucleoside[6,8,9], sugar phosphate[7,8] and nucleotides[6,8,9] with diphosphonate (**3**).

Nucleoside 2'- and 3'-H-phosphonates (**5** and **6**), which are used as valuable intermediates in the synthesis of DNA, are almost separated, as shown in Fig. 6a, in which the UV absorption of each compound is detected. With sulphite (Fig. 6b), nucleoside 2'-H-phosphonate is separated and detected but 3'-H-phosphonate is overlapped with the peak of diphosphonate. No peaks were observed without sulphite (dotted line in Fig. 6b).

Glucose 1-monophosphate (GlclP) and phosphonylated GlclP (**7**) were detected with sulphite, as shown in Fig. 7b. Peaks of GlclP and **7** also appeared without sulphite (dotted line in Fig. 7b) owing to the detection of phosphate groups on both compounds. Sugar phosphate and H-phosphonate, which could not be detected by UV absorption as sown in Fig. 7a, were easily analysed by use of the HPLC–FIA system. The ratio of phosphate to posphonate groups on **7** was found to be *ca.* 1.0 by differentiation of the peak areas of phosphate and phosphonate (**3**) reacts with nucleoside monophospates[8] or diphosphates[9] to form a new class of H-phosphonate analogues of nucleoside polyphosphates (**8** and **9** in Fig. 1). Examples of the separation of such compounds are shown in Figs. 8 and 9.

Fig 6. Elution profiles for adenosine phosphonylated by diphosphonate (**3**) at pH 10 and 20°C for 30 min. **2** = Phosphonate; **5** = adenosine 2'-H-phosphonate; **6** = adenosine 3'-H-phosphonate. Adenosine has disappeared after 30-min incubation with diphosphonate. Eluent: 0.15 *M* KCl + 0.1% Na₄EDTA. Wavelength and meaning of the solid and the dotted lines in (b) as in Fig. 4. Peak numbers correspond to the compound numbers in Fig. 1.

Fig. 7. Elution profiles for glucose 1-phosphate (GlclP) phosphonylated by diphosphonate (**3**) at pH 6 and 70°C for 60 min. **2** = Phosphonate; **7** = phosphonylated GlclP. Eluent: 0.2 *M* KCl + 0.1% Na₄EDTA. Wavelength and meaning of the solid and the dotted lines in (b) as in Fig. 4. Peak numbers correspond to the compound numbers in Fig. 1.

The H-phosphonate analogue (8) of dTDP, dTMP, phosphonate (2) and diphosphonate (3) were resolved completely, as shown in Fig. 8b. The H-phosphonate analogue (8) of dTDP was found to involve nucleoside, phosphate and phosphonate groups from the differentiaton of such groups by means of the HPLC–FIA system as shown in Fig. 8a and b. The ratio of phosphate to phosphonate was confirmed to be *ca.* 1.0.

Fig. 9 shows that all components are separated almost completely. The H-phosphonate analogue of CTP (9 in Fig. 9) also involves nucleoside, phospate, and phosphonate groups, in addition to 8 as described above. The ratio of phosphate to phosphonate, however, is estimated to be *ca.* 2, because 9 has a triphosphate group composed of the phosphate groups and a phosphonate group.

In conclusion, these applications of the HPLC–FIA system clearly demonstrate its potential for the sensitive detection of inorganic, sugar and nucleoside H-phosphonates. Several phospates and H-phosphonates of biological importance were easily separated and detected. The system would be applicable to the simultaneous separation of complex mixtures of inorganic phosphates, inorganic H-phosphonates, biophosphates and "bioH-phosphonates". Such complex mixtures are obtained when a nucleotide reacts with a sugar phosphate, *e.g.*, uridine 5′-triphosphate reacts with GlclP to give uridine 5′-diphosphoglucose (UDPG) and inorganic diphosphate (pyrophosphate)[23,24].

Fig. 8. Elution profiles for thymidine 5′-monophosphate (dTMP) phosphonylated by diphosphonate (3) at pH 6 and 80°C for 4 h. 2 = Phosphonate; 8 = phosphonylated dTMP. Eluent: 0.2 *M* KCl + 0.1% Na$_4$EDTA. Wavelength and meaning of the solid and the dotted lines in (b) as in Fig. 4. Peak numbers correspond to the compound numbers in Fig. 1.

Fig. 9. Elution profiles for cytidine 5′-diphosphate (CDP) phosphonylated by diphosphonate (3) at pH 6 and 70°C for 50 min. 2 = Phosphonate; 9 = phosphonylated CDP. Eluent: 0.3 *M* KCl + 0.1% Na$_4$EDTA. Wavelength and meaning of the solid and the dotted lines in (b) as in Fig. 4. Peak numbers correspond to the compound numbers in Fig. 1.

REFERENCES

1 P. J. Garegg, T. Regberg, J. Stawinski and R. Strömberg, *Chem Scr.*, 25 (1985) 280 and 26 (1986) 59.
2 P. J. Garegg, I. Lindh, T. Regberg, J. Stawinski and R. Strömberg, *Tetrahedron Lett.*, 27 (1986) 4051 and 4055.
3 B. C. Froehler and M. D. Matteucci, *Tetrahedron Lett.*, 27 (1986) 469.
4 I. Lindh and J. Stawinski, *Nucleic Acids Res. Symp. Ser.*, No 18 (1987) 189.
5 B. C. Froehler, *Tetrahedron Lett.*, 27 (1986) 5575.
6 Y. Baba, Y. Yamamoto, N. Yoza and S. Ohashi, *Nucleic Acids Res. Symp. Ser.*, No. 17 (1986) 69.
7 Y. Baba, M. Onoe, T. Sumiyama, M. Tsuhako, N. Yoza and S. Ohashi, *Chem. Lett.*, 1987 (1987) 1389.
8 Y. Yamamoto, Y. Baba, M. Mizokuchi, M. Onoe, T. Sumiyama, M. Tsuhako, N. Yoza and S. Ohashi, *Bull. Chem. Soc. Jpn.*, 61 (1988) 3217.
9 Y. Baba, T. Sumiyama, M. Tsuhako and N. Yoza, *Bull. Chem. Soc. Jpn.*, 62 (1989) 1587.
10 M. Valcarcel and M. D. Luque de Castro, *J. Chromatogr.*, 393 (1987) 3.
11 J. Růžička and E. H. Hansen, *Flow Injection Analysis*, Wiley, New York, 2nd ed., 1988.
12 Y. Baba, in Y. Takashima and N. Yoza (Editors), *Flow Injection Analysis*, Hirokawa, Tokyo, 1989, Ch. 5.
13 H. Hirai, N. Yoza and S. Ohashi, *J. Chromatogr.*, 206 (1981) 501.
14 Y. Baba, N. Yoza and S. Ohashi, *J. Chromatogr.*, 295 (1984) 153.
15 Y. Baba, N. Yoza and S. Ohashi, *J. Chromatogr.*, 318 (1985) 319.
16 Y. Baba, N. Yoza and S. Ohashi, *J. Chromatogr.*, 348 (1985) 27.
17 Y. Baba, Y. Yamamoto, N. Yoza and S. Ohashi, *Bunseki Kagaku*, 34 (1985) 692.
18 Y. Baba, *Thesis*, Kyushu University, Fukuoka, 1986.
19 T. Nakamura, T. Yano, T. Nunokawa and S. Ohashi, *J. Chromatogr.*, 161 (1978) 421.
20 F. Wöhler, *Justus Liebig Ann. Chem.*, 39 (1841) 252.
21 *Gmelin's Handbuch, Schwefel B-II*, Verlag Chemie, Weinheim, 1960, p. 544.
22 N. Yoza and S. Ohashi, *Bull. Chem. Soc. Jpn.*, 37 (1964) 37.
23 H. Hirano, Y. Baba, N. Yoza and S. Ohashi, *Chem. Lett.*, 1986 (1986) 633.
24 N. Yoza, H. Hirano, Y. Baba and S. Ohashi, *Phosphorus Sulfur*, 30 (1987) 605.