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# Analysis of nucleotides by high-performance liquid chromatography with phosphorus-selective detection

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## ABSTRACT

Phosphorus-selective detection based on the post-column molybdenum blue method was developed for the analysis of nucleotides in high-performance liquid chromatography. Orthophosphate generated from nucleotides reacted with molybdate to form a complex, which was detected by a visible absorption detector at 880 nm. Nucleotides were separated in the reversed-phase ion-pair mode, and operating conditions which influence the signal intensity were examined. Detection limits of 0.2–0.4 nmol per 12 $\mu$l were obtained for the examined nucleotides. The system was applied to the analysis of nucleotides in fish tissues.

## INTRODUCTION

Nucleotides are often analyzed by high-performance liquid chromatography (HPLC) with UV detection. Ion-exchange [1], reversed-phase[2–8] and reversed-phase ion-pair chromatography [9,10] are the most commonly used methods. Nucleotides contain a mono-, di- or triphosphate group in the molecule, and there exist many species resulting from combination with different nucleosides and the position of the attached phosphate group. In addition, interferences from matrices make the analysis more complicated. One of the strategies for solving this problem is to develop a selective detection method for the nucleotides.

Complexation between orthophosphate ($PO_4^{3-}$) and molybdate, *i.e.*, the molybdenum blue method, has been utilized for the selective determination of phosphorus in various samples by means of an off-line method[11], flow-injection analysis [12–14] and continuous-flow analysis [15]. In these methods absorbance of the complex at a visible wavelength is measured to determine total phosphorus. Because it is orthophosphate that forms a complex with molybdate in the molybdenum blue method, condensed phosphoric acid and organophosphorus compounds should be decomposed to generate orthophosphate before the complexation. If the molybdenum blue

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colorimetry can be combined with a separation method, phosphorus compounds can be specified.

In this paper molybdenum blue colorimetry was combined with HPLC for the selective detection of nucleotides. The assembled system was applied to the analysis of nucleotides in fish tissues.

## EXPERIMENTAL

### *Apparatus*

A block diagram of the assembled system is shown in Fig. 1. An LC-6AD HPLC pump (No. 2) (Shimadzu, Kyoto, Japan) and a Minipuls 2 peristaltic pump (No. 16) (Gilson, Villiers-le-Bel, France) were used to deliver the mobile phase and the reagents, respectively. The line filter (No. 3) was composed of a 10 mm × 4.6 mm I.D. column packed with 30-$\mu$m porous silica (Develosil; Nomura Chemical, Seto, Japan). The loop injector (No. 4) was assembled by using a Model 7000 switching valve (Rheodyne, Cotati, CA, U.S.A.) in the laboratory, and the loop volume was 12 $\mu$l. Develosil ODS-5 (5 $\mu$m particle diameter, 250 mm × 4.6 mm I.D.; Noruma Chemical) was employed as the separation column (No. 5). The effluent from the separation column was monitored by a Uvidec-100 V UV spectrophotometer (No. 6) (Jasco, Tokyo, Japan) at 250 nm, followed by post-column reaction for the phosphorus-selective detection.

The eluent was first mixed with 4% (w/v) potassium peroxodisulfate aqueous solution (No. 14) and passed through the 8 m × 0.5 mm I.D. PTFE reaction coil (No. 10) heated in an EMG-1 aluminium block bath (No. 9) (Eyela, Tokyo, Japan), in order for the samples to undergo oxidative decomposition, in which orthophosphate was formed from nucleotides. The orthophosphate formed was then converted to the phosphorus–molybdenum complex by reaction with 2.5% (w/v) ammonium molybdate acidic solution (color-forming reagent; No. 15) in the 5 m × 0.5 mm I.D. PTFE reaction coil (No. 11) at room temperature. The color-forming reagent contained 0.36% (w/v) L-ascorbic acid and 1.5 *M* sulfuric acid. The molybdenum blue complex was monitored by a Uvidec-100 IV spectrophotometer (No. 12) (Jasco) at 880 nm. The flow cell of the latter detector as well as the hydraulic line for the post-column detection were metal-free because the reagents were strongly acidic.

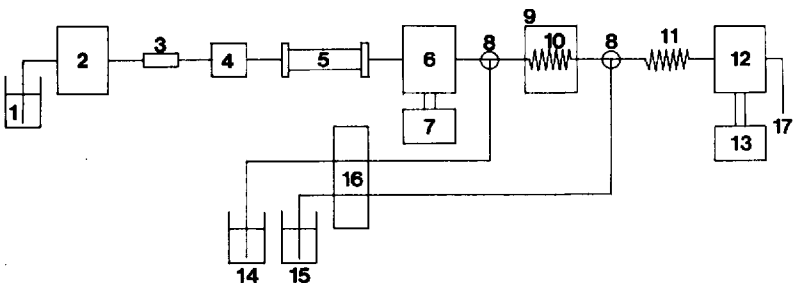


Fig. 1. Block diagram of the apparatus. 1 = Mobile phase; 2 = HPLC pump; 3 = line filter; 4 = loop injector; 5 = separation column; 6 = UV detector; 7 = chart recorder; 8 = T-joint; 9 = aluminum block bath; 10 = reaction coil (8 m × 0.5 mm I.D.); 11 = reaction coil (5 m × 0.5 mm I.D.); 12 = visible absorption detector; 13 = integrator; 14 = oxidative reagent; 15 = color-forming reagent; 16 = peristaltic pump; 17 = waste.

*Reagents*

All the reagents were of reagent grade and obtained from Wako (Osaka, Japan) or Tokyo Chemical Industry (Tokyo, Japan), unless otherwise stated. Purified water was prepared by using a Milli-Q reagent water system (Japan Millipore, Tokyo, Japan). The following nucleotides were obtained from Wako: adenosine 5′-monophosphate (AMP), adenosine 5′-diphosphate (ADP), adenosine 5′-triphosphate (ATP), cytidine 5′-monophosphate (CMP), guanosine 5′-monophosphate (GMP), inosine 5′-monophosphate (IMP), thymidine 5′-monophosphate (TMP) and uridine 5′-monophosphate (UMP).

Fish samples were prepared as follows: approximately 8 g of fish tissues were homogenized by adding 25 ml of 1 *M* perchloric acid, followed by centrifugation at 1600 *g* for 5 min. A 5-ml volume of the supernatant was taken, and its pH was adjusted to 6.5 with 1-*M* potassium bicarbonate. A portion of the supernatant was finally injected into the HPLC system.

The HPLC mobile phase was a mixture of a buffer and acetonitrile. The buffer contained tetrabutylammonium chloride, 20 m*M* boric acid, 10 m*M* sodium borate and 5 m*M* citric acid. The pH of the buffer was finally adjusted to 5.4 with sulfuric acid.

## RESULTS AND DISCUSSION

Organophosphorus compounds must be decomposed to generate orthophosphate before complexation in molybdenum blue colorimetry, and thus several parameters could affect the signal intensity, for example mobile phase components, flow-rates of the mobile phase and the reagents, reaction conditions, etc. The effects of these parameters on the signal intensity were examined without the separation column. In reversed-phase ion-pair chromatography, the mobile phase is commonly composed of ion-pair reagent, buffer solution and organic solvent. In this work tetra-

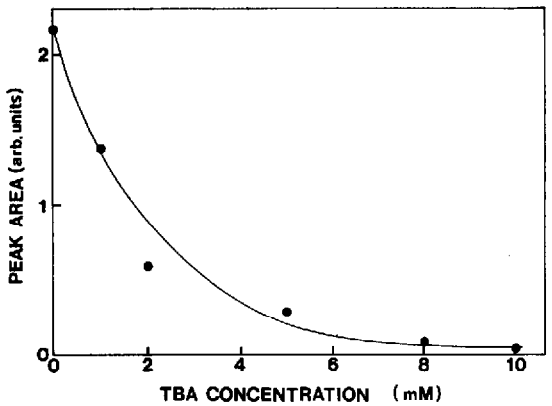


Fig. 2. Effect of the concentration of the ion-pair reagent in the eluent on the signal intensity. Eluent: borate buffer (pH 5.4) containing tetrabutylammonium chloride (TBA) and 5 m*M* citric acid–acetonitrile (98:2). Flow-rate of the eluent: 0.7 ml/min. Flow-rate of the oxidative reagent and color-forming reagent: 0.1 ml/min. Temperature of the aluminum block bath: 128°C. Solute: ATP. Wavelength of detection: 880 nm.

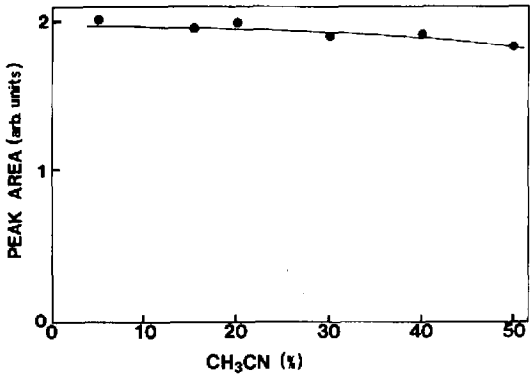


Fig. 3. Effect of acetonitrile concentration in the eluent on the signal intensity. Operating conditions as in Fig. 2 except the eluent composition (mixture of acetonitrile and borate buffer at pH 5.4 containing 1 m*M* TBA and 5 m*M* citric acid). Solute: ATP.

butylammonium chloride was used as the ion-pair reagent, and borate and acetonitrile were used as the buffer and the organic solvent, respectively.

Fig. 2 demonstrates the effect of the concentration of the ion-pair reagent in the eluent on the signal intensity. The peak heights observed without the separation column are plotted in the figure. The smaller is the concentration of the ion-pair reagent, the higher the signal intensity observed. In the reversed-phase ion-pair mode, the retention time of analytes decreases with decreasing ion-pair reagent concentration, which can be compensated for by decreasing acetonitrile concentration in the mobile phase. In the following experiments, the concentration of tetrabutylammonium chloride was kept constant at 1 m*M*.

Fig. 3 illustrates the effect of acetonitrile concentration in the eluent on the signal intensity. The results indicate that the effect of the acetonitrille concentration is not very significant up to 50% (v/v). Isocratic elution was carried out for the sep-

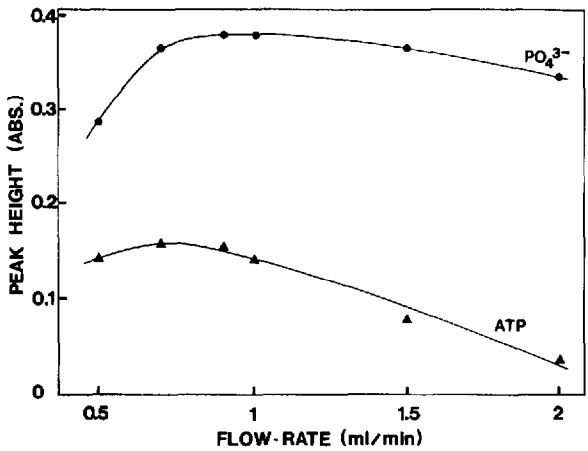


Fig. 4. Effect of the flow-rate of the mobile phase on the signal intensity. Operating conditions as in Fig. 2 except the solutes. (orthophosphate and ATP).

aration of nucleotides, and the mobile phase containing 1 m*M* tetrabutylammonium chloride and 2% (v/v) acetonitrile was mostly employed. The ionic strength of the mobile phase was adjusted with borate and citric acid as described in the Experimental section, and the pH of the mobile phase was adjusted to 5.4 with sulfuric acid.

The effect of the flow-rate of the mobile phase on the signal intensity is shown in Fig. 4. The flow-rates of both oxidative and color-forming reagents were kept constant at 0.1 ml/min. A flow-rate of approximately 0.7 ml/min was optimum for ATP, while around 1 ml/min was optimum for orthophosphate. The peak height decreases with increasing flow-rate, which is because the dispersion of the analyte in the hydraulic line becomes more significant and the concentration of the ion-pair reagent in the mixed solution increases.

The oxidative decomposition temperature remarkably influenced the signal intensity, especially for organophosphorus compounds. Fig. 5 shows the dependence of the signal intensity on the oxidative decomposition temperature. Potassium peroxodisulfate was employed as the oxidative reagent. The signal intensity increased with increasing decomposition temperature for ATP, while the signal was nearly constant for orthophosphate. The present system allowed heating to temperatures up to 135°C. At higher temperatures, the air bubble formed interfered with detection. The bubble formation could be eliminated by applying pressure from the downstream side of the flow cell of the detector, but the peristaltic pump employed in turn did not work well at several atmospheric pressures. Therefore, the oxidative decomposition was carried out at approximately 130°C. In addition, even without oxidation by potassium peroxodisulfate, the monophosphate-type nucleotides examined in this work (AMP, CMP, GMP, IMP, TMP and UMP) could be detected to some extent, *e.g.*, 40–50% of the signals observed using the oxidative decomposition process could be detected. However, diphosphate-type and triphosphate-type nucleotides could not be detected without the oxidative reaction process.

The effect of the flow-rate of the color-forming reagent on the signal intensity was examined. The flow-rate of the mobile phase was kept constant at 0.7 ml/min. The reagent employed was 1.5 *M* sulfuric acid solution containing 2.5% ammonium

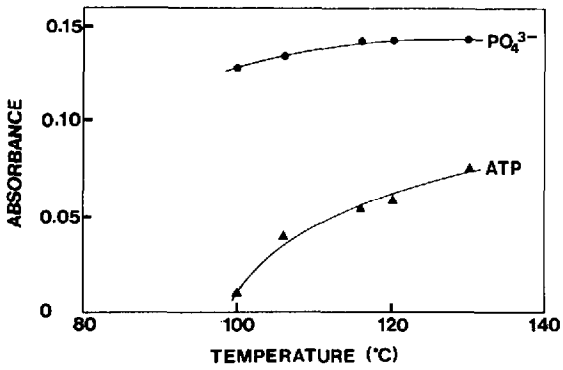


Fig. 5. Dependence of the signal intensity on the oxidative decomposition temperature. Operating conditions as in Fig. 2 except the solutes. (orthophosphate and ATP).

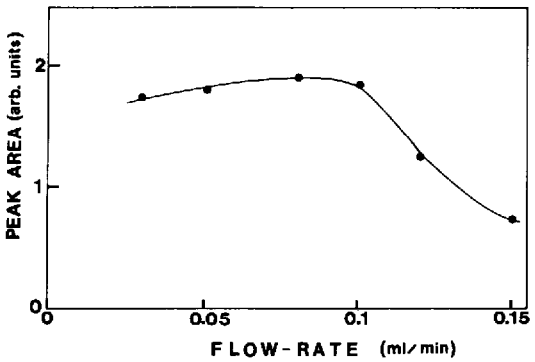


Fig. 6. Effect of the color-forming reagent on the signal intensity. Operating conditions as in Fig. 2 except the flow-rate of the color-forming reagent.

molybdate and 0.36% L-ascorbic acid. A flow-rate of approximately 0.08–0.1 ml/min gave a higher signal intensity, as shown in Fig. 6.

Using the compromise operating conditions determined by the above experiments, an artificial mixture of eight nucleotides was separated in the reversed-phase ion-pair mode, as shown in Fig. 7. The operating conditions are described in the figure legend. The wavelength of detection was 880 nm, and 0.7 12 nmol of the components were injected using a 12-$\mu$l loop injector. The detection limits for the examined nucleotides were 0.2–0.4 nmol under the operating conditions shown in Fig. 7.


Fig. 7. Separation of an artificial mixture of nucleotides. Separation column: Develosil ODS-5, 250 mm × 4.6 mm I.D. Mobile phase: borate buffer (pH 5.4) containing 1 m*M* tetrabutylammonium chloride and 5 m*M* citric acid–acetonitrile (98:2). Flow-rate of the mobile phase: 0.7 ml/min. Flow-rates of the oxidative reagent and the color-forming reagent: 0.1 ml/min. Temperature of the aluminium bath: 128 °C. Wavelength of detection: 880 nm. Solutes: 12.2 nmol IMP; 4.9 nmol AMP; 4.9 nmol CMP; 4.7 nmol ADP; 4.4 nmol ATP; 0.7 nmol TMP; 0.7 nmol UMP; and 1.5 nmol GMP.

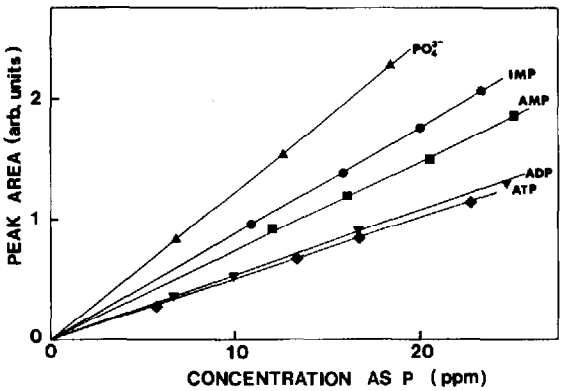


Fig. 8. Calibration graphs for nucleotides. Operating conditions as in Fig. 7.

Fig. 8 shows calibration graphs for orthophosphate, IMP, AMP, ADP and ATP. The peak areas are plotted *versus* the concentration of phosphorus in ppm. The results show that the conversion of the phosphate group of the nucleotides to orthophosphate is not perfect, *e.g.* 72% for IMP, 61% for AMP, 45% for ADP and 43% for ATP. A single calibration graph should be applicable to all the nucleotides if the conversion of all the phosphorus forms to orthophosphate is complete. The detection limits achieved by the molybdenum blue method were higher than those obtained by detection of inherent UV absorption. This is because the noise level of the phos-

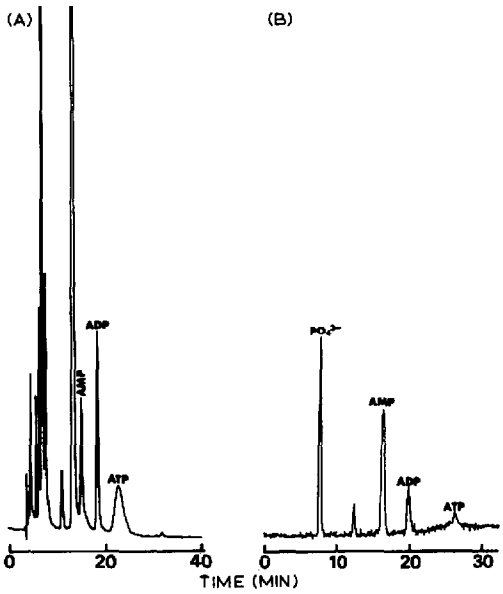


Fig. 9. (A) UV and (B) phosphorus-selective detection of components contained in fish tissues. Operating conditions as in Fig. 7 except the sample (tuna) and detection wavelengths. (A 250 nm; B, 880 nm).

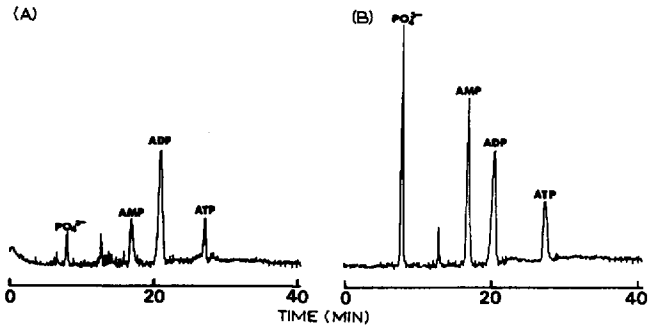


Fig. 10. Phosphorus-selective detection of components in fish tissues. Operating conditions as in Fig. 7 except the samples: (A) porgy; (B) yellowtail.

phorus-selective detection was much higher than that of UV detection owing to the pulsation in the post-column mixing. Howevere, UV detection is not selective, and thus matrices make the analysis of real samples more difficult.

The present system was applied to the analysis of nucleotides contained in raw fish tissues. Fig. 9 demonstrates UV (Fig. 9A) and phosphorus-selective detection (Fig. 9B) of components contained in tuna. The UV detection shows many peaks other than nucleotides, while the phosphorus detection shows a simple chromatogram. The latter detection method allows determination of the nucleotides without problems caused by the interferences from matrices. Orthophosphate, AMP, ADP and ATP could be identified from the retention times of the standard solutes, while the second peak could not be identified. By using the calibration graphs shown in Fig. 8, AMP, ADP and ATP concentrations were determined to be 5.4, 1.7 and 0.6 nmol respectively. The relative standard deviations of the peak area for five measurements of the reference analytes were 0.41 to 1.8%.

Applications to other fish samples, porgy and yellowtail, are shown in Fig. 10. The same components as in Fig. 9 are identified. For the porgy sample, amounts of 2.0 nmol AMP, 4.7 nmol ADP and 1.6 nmol ATP were observed, while 4.9 nmol AMP, 4.0 nmol ADP and 1.9 nmol ATP were observed for the yellowtail sample. The results are listed in Table I. The concentrations of the observed nucleotides were 0.3–3 μmol/g for the the examined raw fish tissues.

TABLE I

NUCLEOTIDES CONTAINED IN RAW FISH TISSUES

| Fish | Concentration[a] (μmol/g) | | |
|---|---|---|---|
| | AMP | ADP | ATP |
| Tuna | 2.2 | 0.71 | 0.26 |
| Porgy | 1.0 | 2.4 | 0.81 |
| Yellowtail | 2.6 | 2.1 | 0.98 |

[a] Based on fresh weight of sample.

## CONCLUSION

Phosphorus-selective detection based on the molybdenum blue method could be effectively applied to the analysis of nucleotides, but its detection limits were higher than the detection of inherent UV absorption of the analytes. This is because the former detection method has a higher noise level resulting from post-column mixing. If the pulsation could be eliminated, the sensitivity of the phosphorus-selective detection system would be improved. The present system will be applicable to the selective detection of other phosphorus compounds.

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