

Control of the Contaminant Level for Determination of  $\text{Al}^{3+}$  Using 8-Quinolinol by  
High-Performance Liquid Chromatography with Fluorescence Detection

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## Abstract

We developed a technique controlling contaminant  $\text{Al}^{3+}$  level using a combination of kinetics and thermodynamics at a pre-column derivatizing step in HPLC. The technique involves modifying a conventional HPLC of 8-quinolinol (Ox) complex with  $\text{Al}^{3+}$ . The contaminant suppressing reagents, dihydroxyazobenzene (DHAB) and 1,2,3-trihydroxybenzen (pyrogallol) were added to the Ox and pH buffer solutions to convert contaminant  $\text{Al}^{3+}$  in these solutions to inactive complexes. The  $\text{Al}^{3+}$  in the sample selectively formed Ox complexes with fast kinetics. After that, the labeled fluorescent complexes in the resultant metastable state were separated in the HPLC. This technique successfully suppressed contamination by a factor of 17. This method allowed for an improvement in the detection limits and also provided a stable blank.

**Keywords.** Contamination suppression, pre-column derivatization, aluminum, dihydroxyazobenzene, pyrogallol, 8-quinolinol, fluorescent detection

## 1. Introduction

The trace analysis of metal ions is difficult and troublesome if contamination of the objective metal ions occurs from the reagent solutions, the instruments employed or the atmosphere. Especially, the determination of ubiquitous metal ions at higher than natural concentrations, such as  $\text{Ca}^{2+}$ ,  $\text{Zn}^{2+}$  and  $\text{Al}^{3+}$  etc., is substantially influenced by experimental conditions. In fact, the analysis of  $\text{Al}^{3+}$  lower than  $\text{ng ml}^{-1}$  in HPLC tends to be accompanied by contaminant  $\text{Al}^{3+}$ , which is observed as a distinct blank peak. Moreover, since the contaminant level often fluctuates, the stabilization of the blank level is the key to obtaining highly reproducible measurements. In spite of the importance of controlling the contamination level, there are few reports on techniques to effectively avoid contamination interference except for the purification of reagents and the employment of a clean room.

Recently, we reported a new method to control the contamination from the reagents used [1]. The concept is to convert contaminant  $\text{Al}^{3+}$  to an inactive species and to selectively derivatize the analyte  $\text{Al}^{3+}$  in a sample to a fluorescent complex based on the difference of the complex formation kinetics in the pre-column derivatization step in HPLC. The procedure is very simple. It requires only the addition of blocking agents to all the solutions employed, i.e. the pH buffer, the fluorescent labeling agent solution and the mobile phase. To make up such a contaminant-free reaction system, specific conditions need to be met. 1) The stability constant of the  $\text{Al}^{3+}$  complexes with blocking ligand (B),  $K_B$ , has to be sufficiently larger than those of the complexes with the labeling agent (L) and the pH buffer agent,  $K_L$  and  $K_{\text{Buffer}}$ , respectively ( $K_B \gg K_L, K_{\text{Buffer}}$ ).

1 It must be large enough to form the complexes of all the contaminant  $\text{Al}^{3+}$  ions with B in the  
2 stock solutions. In this method, the contaminant metal ions are completely converted to inactive  
3 Al-B species in the stock solutions. 2) The formation rate constant of the labeled complex has to  
4 be sufficiently larger than that of the blocking complex ( $k_L \gg k_B$ ), so that  $\text{Al}^{3+}$  ions in a sample  
5 predominantly bind with labeling agents with fast kinetics when the sample is mixed with a  
6 labeling agent and a pH buffer solution at a pre-column derivatizing step. 3) Each of the  
7 dissociation rate constants, that of the labeled complex ( $\text{Al-L} \rightarrow \text{Al}^{3+} + \text{L}$ ;  $k_L$ ) and the blocking  
8 complex ( $\text{Al-B} \rightarrow \text{Al}^{3+} + \text{B}$ ;  $k_B$ ), and ligand-exchange rate constant ( $\text{Al-L} + \text{B} \rightarrow \text{Al-B} + \text{L}$ ;  $k_{\text{ex}}$ ),  
9 has to be small enough to keep the labeling agent forming the complex only with sample  $\text{Al}^{3+}$  and  
10 the blocking agent forming a complex only with contaminant  $\text{Al}^{3+}$  in the prepared sample solution  
11 containing L, B, the pH buffer, the analyte  $\text{Al}^{3+}$  and contaminant  $\text{Al}^{3+}$ . When the requirements are  
12 all satisfied, all the contaminant  $\text{Al}^{3+}$  remains in the complex with B until the measurements are  
13 taken, and only the sample  $\text{Al}^{3+}$  is converted to the L complex. It is noted the slowly proceeding  
14 ligand exchange reaction ( $\text{Al-L} + \text{B} \rightarrow \text{Al-B} + \text{L}$ ,  $K_{\text{ex}} = K_B/K_L > 1$  because  $K_B \gg K_L$ ,  $K_{\text{Buffer}}$ )  
15 makes this state metastable. After that, the analytes are separated and detected in a conventional  
16 manner in the HPLC. In principle, the control of contamination using such procedures is not  
17 possible using instrumental analytical methods such as ICP-MS and AES, because the  
18 contaminant metal ions in the reagents employed are atomized and ionized together with sample  
19 metal ions in these methods. In other words, using these methods, it is not possible to  
20 discriminate between the sample and contaminant metal ions.

For a chemical reaction system which satisfied all the abovementioned requirements, calcein and *o,o'*-dihydroxyazobenzene (DHAB) were employed as the labeling and blocking agents, respectively, in our previous work. This system worked successfully: the contamination of  $\text{Al}^{3+}$  was suppressed to  $10^{-9}$ - $10^{-10}$  mol dm<sup>-3</sup> level in the HPLC and capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) provided low detection limits at low pg ml<sup>-1</sup> levels [1, 2].

We report here an application of this technique to a commercially available system for the specific determination of  $\text{Al}^{3+}$  in HPLC with fluorescence detection. A reversed-phase (RP) HPLC of tris(8-quinolinolato)aluminum(III) complex ( $\text{Al}(\text{Ox})_3$ ) with fluorescent detection was selected for this study. This is a system well known for its high selectivity, sensitivity and simplicity, and one which is applied to practical quality control in the pharmaceutical industry [3-6]. The HPLC system using the  $\text{Al}(\text{Ox})_3$  complex was developed by Sato et al.[7] and the principle of HPLC for detecting metal ions in a series of works by Yotsuyanagi et al. was named the Kinetically Differentiation (KD) mode HPLC, in which only kinetically stable metal complexes were specifically detected [8-14]. This system was found to be simple, tough and robust. It is considered suitable for practical use with respect to its selectivity and sensitivity. However, when the 8-quinolinol method for  $\text{Al}^{3+}$  is carried out, the contamination problem still occurs, and the inconvenience of having to measure trace level  $\text{Al}^{3+}$  arises. We selected DHAB and pyrogallol (abbreviated as Py or  $\text{H}_3\text{Py}$ ) as blocking agents to suppress the contamination in the pH buffer and Ox solutions after taking the thermodynamics and kinetics into consideration

(vide infra). Our goal in this investigation was to develop an easier handling technique with regard to the suppression and stabilization of the contamination levels of  $\text{Al}^{3+}$  in the 8-quinolinol method without any purification.

## 2. Experimental

### 2.1. Apparatus.

The used HPLC setup consisted of a Model LC10-AD pump unit, a Model RF-10AXL fluorescence detector (Shimadzu, Kyoto, Japan), and a Rheodyne Model 7725 sample injection valve with a 20  $\mu\text{L}$  loop. A Shimadzu C-R5A Chromatopack was employed as a recorder. In order to avoid any serious contaminant  $\text{Al}^{3+}$ , per-fluoro-alkoxy (PFA) laboratory wares were exclusively employed. The analytical reversed-phased phenyl column used was a Develosil Shinal Column (35 mm  $\times$  4.6 mm I. D.) (Nomura Chemical, Seto, Japan).

### 2.2. Chemicals.

The fluorescent labeling reagent, 8-quinolinol (Ox), was dissolved in deionized water (Milli-Q SP. TOC. system, Millipore, Billerica, MA, USA) to give a concentration of 0.2 mol  $\text{dm}^{-3}$  with an appropriate amount of 20% HCl (Tama Chemicals, Kawasaki, Japan) at pH 2.0. In the Ox stock solution, the blocking reagent, 1,2,3-trihydroxybenzene (pyrogallol), purchased from Kishida Chemical (Osaka, Japan), was dissolved to give a concentration of  $1 \times 10^{-5}$  mol  $\text{dm}^{-3}$ . The 2.5 mol  $\text{dm}^{-3}$  pH buffer solutions of *N,N*-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) and

Tris(2-Amino-2-hydroxymethyl-1,3-diol (Tris) (Wako Pure Chemical Industries, Osaka, Japan) were used at pH 7.5, with the pH adjusted by the addition of tetramethylammonium hydroxide (TAMAPURE-AA-10 quality,  $\text{Al} < 10 \text{ ng ml}^{-1}$ ) (Tama Chemicals) and concentrated HCl, respectively. Another blocking agent, *o,o'*-dihydroxyazobenzene (DHAB) (analytical grade, Tokyo Kasei Kogyo, Tokyo, Japan) was dissolved in the pH buffer solutions to the desired concentrations (typically  $5 \times 10^{-6} \text{ mol dm}^{-3}$ ). The standard solutions of aluminum ions were prepared by dissolving aluminium nitrate nonahydrate salts (99.9 % purity, Wako Pure Chemical Industries) in deionized water with a few drops of concentrated hydrochloric acid, and set to pH 2.

### 2.3 HPLC Procedure.

The stock solutions of Ox ( $0.2 \text{ mol dm}^{-3}$ ) including  $1.0 \times 10^{-5} \text{ mol dm}^{-3}$  pyrogallol at pH 2, and the pH buffers, Tris, BES and HEPES ( $2.25 \text{ mol dm}^{-3}$  at pH 7.5), including  $5.0 \times 10^{-6} \text{ mol dm}^{-3}$  DHAB were prepared in order to mask the contaminant  $\text{Al}^{3+}$ . These solutions were left in the dark over night at room temperature in order to reach an equilibrium state, in which all contaminant  $\text{Al}^{3+}$  in stock solutions formed complexes with DHAB and pyrogallol. Fifty  $\mu\text{L}$  of Ox and 400  $\mu\text{L}$  of the pH buffer solution containing the blocking agents were successively added to 150  $\mu\text{L}$  of the sample solution. After allowing the mixture to stand for 10 minutes at room temperature, an aliquot ( $\sim 500 \mu\text{L}$ ) of the mixture was loaded on an injection valve (20  $\mu\text{L}$  loop) for HPLC analysis. In the mixture, the concentrations of the Ox, pH buffers, DHAB and pyrogallol were

made as  $1.67 \times 10^{-2}$ ,  $1.5$ ,  $3.3 \times 10^{-6}$  and  $8.3 \times 10^{-7}$  mol dm<sup>-3</sup>, respectively.

Typical HPLC conditions of the mobile phase were prepared according to a previous paper (a 2-propanol-water solution including pH buffer and SDS) [15]. The mobile phase solution purchased from Shino Test Co. was employed as it was. The flow rate was set at 1 ml min<sup>-1</sup>. Fluorescence detection was carried out with excitation and emission wavelengths of 370 nm and 504 nm, respectively. None of the experiments were done in a clean room.

### 3. Results and Discussion

#### 3.1. Thermodynamic calculation and setting the concentration of blocking reagents

Since the most thermodynamically stable B (the blocking agent) complex was necessary for each solution employed, the concentration of B was set after estimating the distribution of Al species in the equilibrium state. The complexing agents, DHAB and Py, were selected as B reagents since their thermodynamic stabilities with Al<sup>3+</sup> are very high. The stability constants for Al-(Ox)<sub>3</sub>, Al-(Py)<sub>3</sub> and Al-(DHAB)<sub>2</sub> and acid dissociation constants for each ligand were reported previously ( $\beta_{\text{Al(Ox)}_3} = 10^{22}$  [16],  $K_{\text{Al-Ox}} = 10^{11.9}$ ,  $K_{\text{a1, Ox}} = 10^{5.14}$ ,  $K_{\text{a2, Ox}} = 10^{9.74}$  [17,18],  $\beta_{\text{Al-(HPy)}_3} = 10^{58.9}$ ,  $\beta_{\text{Al-(HPy)}_2} = 10^{44.5}$ ,  $K_{\text{Al-HPy}} = 10^{24.5}$ ,  $K_{\text{a1, Py}} = 10^{-8.92(-8.94)}$ ,  $K_{\text{a2, Py}} = 10^{-10.97(-11.08)}$ ,  $K_{\text{a3, Py}} = 10^{-14.00}$  [18-20],  $\beta_{\text{Al-(DHAB)}_2} = 10^{29.1}$ ,  $K_{\text{Al-DHAB}} = 10^{16.4}$ ,  $K_{\text{a1, DHAB}} = 10^{-8.20}$  and  $K_{\text{a2, DHAB}} = 10^{-11.6}$ ) [22, 23]. Here, it is noted that the stability constants of Py are those of the Al<sup>3+</sup> complex with monoprotonated Py (HPy<sup>2-</sup>).

To suppress contaminant Al in the pH buffer solution, DHAB was employed as a blocking

1 agent. The Py was not suitable as a B reagent for the pH buffer stock solution since it oxidizes at  
2 neutral pHs (the pH value of the buffer solutions was set at 7.5). Since the pH buffer agents used  
3 (Tris, BES and HEPES) probably have a considerably smaller stability constant with  $\text{Al}^{3+}$  than  
4 DHAB, it was expected that almost all the  $\text{Al}^{3+}$  ions would form DHAB complexes ( $K_B \gg K_L$ ;  
5 requirement 1 in Introduction). Regarding the second requirement ( $k_L \gg k_B$ ), it was reported that  
6 both the formation and dissociation processes of  $\text{Al}-(\text{DHAB})_2$  complex were very slow [1, 22, 24,  
7 25], thus satisfying the requirement. Although DHAB is a good potential candidate for the pH  
8 buffer solution, the degree of suppression cannot be theoretically calculated since there are no  
9 data for the thermodynamics of  $\text{Al}$ -Tris, -BES and -HEPES complexes. Chemical suppression  
10 actually had to be demonstrated by experiments (vide infra).

11 In contrast to pH buffer solutions, a somewhat theoretical estimation was possible for  $\text{Al}^{3+}$   
12 suppression in the Ox stock solution using stability constants. It is expected that DHAB cannot  
13 effectively block the contaminant  $\text{Al}$  in the Ox solution since the conditional thermodynamic  
14 stability constants at pH 2 for  $[\text{Al-DHAB}]^+$  and  $[\text{Al}-(\text{DHAB})_2]^-$  are very small, calculated as  
15  $K'_{\text{Al-DHAB}} = 10^{0.6} \text{ mol}^{-1} \text{ dm}^3$  and  $\beta'_2{}_{\text{Al}-(\text{DHAB})_2} = 10^{-2.5} \text{ mol}^{-2} \text{ dm}^6$ , respectively. With these very low  
16 stabilities, it is impossible to block the  $\text{Al}$  contaminant over 99% until  $10^{1.4} \text{ M}$  DHAB is added;  
17 therefore, it is not suitable for practical use. Pyrogallol (Py) was selected as a B agent for the Ox  
18 stock solution. Py is a stronger chelator of  $\text{Al}^{3+}$  than DHAB even at low pH conditions, which  
19 makes it suitable for the Ox solution at low pH. At pH 2 in the Ox stock solution, the conditional  
20 stability constants,  $\beta'_3$  and  $K'_1$  for  $\text{Al}$ -Ox species were calculated as  $10^{-10.1}$  and  $10^{1.02}$ , and  $\beta'_3$   $\beta'_2$

1 and  $K_1'$  for Al-HPy species were  $10^{11.23}$ ,  $10^{11.23}$  and  $10^{8.61}$ , respectively. Judging from these  
2 constants, the Al-HPy species was clearly predominant over Al-Ox species. When more than  $1 \times$   
3  $10^{-5}$  M of Py was added to the Ox stock solution (0.2 M) at pH 2, over 99.9 % of  $\text{Al}^{3+}$  existed as  
4 Py complexes, including Al-(HPy)<sub>2</sub> (with a distribution of approximately 80%), and Al-HPy  
5 (approx. 20%); the concentration of the Al-Ox complex was lower than 0.1 % (the ratio of  
6  $[\text{Al-HPy}]/[\text{Al-Ox}] = 10^{6.89}$ ). Under these conditions, contaminant  $\text{Al}^{3+}$  in the Ox stock solution  
7 was completely blocked in order to meet the first requirement. The concentration of Py in the Ox  
8 solution was set at  $1.0 \times 10^{-5}$  mol dm<sup>-3</sup>. The formation of mixed-ligand complexes such as  
9 Al-Ox-HPy is possible. Since, unfortunately, there is no thermodynamic data for those reactions,  
10 the formation of the mixed-ligand complexes is not taken into consideration in this study.  
11 However, it can be assumed that those mixed-ligand species have an inert nature due to the  
12 intrinsic characteristics of  $\text{Al}^{3+}$ , and the suppression of the contaminant Al is probably made by  
13 also the mixed-ligand complexes. The results supported this assumption (vide infra).

14 For a pre-column derivatizing step at pH 7.5, the pH buffer solution with DHAB was mixed  
15 with the Ox solution with Py, in which all contaminant Al formed the Al-B complexes (B =  
16 DHAB and Py). In this state, Ox can be assumed to predominantly form its Al complex with  
17 faster kinetics than Al-DHAB and Al-Py complexes ( $k_{\text{Al-(Ox)}_3} \gg k_{\text{Al-(HPy)}_3}$ ,  $k_{\text{Al-buffer}}$ ). It was  
18 reported that the formation of Al-(Ox)<sub>3</sub> complex was so fast that it completed the reaction within  
19 ten minutes under pseudo-first order conditions with a large excess of 1 mM Ox at pH 7.5 [4,5,7].  
20 Meanwhile, the formation rate constant of the Al-DHAB complex was determined as  $k_B = 69$

1  $s^{-1}M^{-1}$  [1]. The concentration of DHAB in a mixed sample solution set in the present work was  $3.3$   
2  $\times 10^{-6}$  M. Under these conditions, it took no less than 2.4 hours for 99 % of the Al-DHAB  
3 complex formation reaction to take place according to our calculations. These slow kinetics  
4 enable Al-(Ox)<sub>3</sub> to be the predominant complex formed in the timescale of the experiment.  
5 Therefore, the complex formation of DHAB with sample Al<sup>3+</sup> did not take place. When Py was  
6 used in the mixed solution, even though there is no data for the formation rate constant of the Al<sup>3+</sup>  
7 complex, it was experimentally demonstrated by the high recovery of sample Al<sup>3+</sup> that the added  
8 pyrogallol did not affect the complexation of Ox with sample Al<sup>3+</sup> at the pre-column process (see  
9 Results and Discussion).

10 Finally, it can also be assumed that no ligand-exchange reactions took place in the prepared  
11 sample solution and that the concentrations of Al species, therefore, did not change, i.e. all the  
12 contaminant Al<sup>3+</sup> remained in preformed complexes with DHAB or Py, and all the sample Al<sup>3+</sup>  
13 remained free to form complexes with Ox in precolumn sample solution. Four ligand-exchange  
14 reactions would normally be expected to take place: reaction a,  $Al-(DHAB)_2 + 3Ox \rightarrow Al-(Ox)_3 +$   
15  $2DHAB$ ;  $K_{ex,DHAB}$  and reaction b,  $Al-(Py)_3 + 3Ox \rightarrow Al-(Ox)_3 + 3Py$ ;  $K_{ex,Py}$  and the reverse  
16 reactions, reaction c;  $K_{-ex,DHAB}$  and reaction d;  $K_{-ex,Py}$ . However, reactions a, c and d would not  
17 have taken place because Al-(DHAB)<sub>2</sub> and Al-(Ox)<sub>3</sub> were quite kinetically stable species with  
18 respect to dissociation and ligand-exchange reactions, as reported previously [1, 4, 26]. With  
19 respect to the kinetic stability of the Al-(Ox)<sub>3</sub> complex, it should be noted that the complex was  
20 not decomposed even under the strong driving force of dissociation to coexist with  $10^{-4}$  M EDTA.

Whether or not reaction b progressed can be assessed by considering the equilibrium constants.

The conditional stability constants at pH 7.5, at which the pre-column reaction was carried out,

are  $\beta_{\text{Al(Ox)}_3} = 10^{15.4}$  and  $\beta_{\text{Al(HPy)}_3} = 10^{44.33}$ . Therefore, the ligand exchange equilibrium constants

at pH 7.5 are represented as

$$K_{\text{ex,Py}} = \frac{[\text{Al-(Ox)}_3][\text{Py}]^3}{[\text{Al-(Py)}_3][\text{Ox}]^3} = 10^{-28.83} \quad (1)$$

Judging from the thermodynamic stability constant, reaction b cannot take under the experimental

conditions ( $[\text{Ox}] = 0.0167 \text{ M}$  and  $[\text{Py}] = 8.3 \times 10^{-7} \text{ M}$ ).

Thus, almost all the requirements for the suppression of contaminant Al were satisfied.

However, whether the  $\text{Al-(Ox)}_3$  complex formation predominated over the  $\text{Al-(HPy)}_3$  complex

( $k_{\text{Al-(Ox)}_3} \gg k_{\text{Al-(HPy)}_3}$ ) at the precolumn derivatization step, and whether the contaminant could be

successfully suppressed by DHAB in the pH buffer solution had to be confirmed experimentally.

Furthermore, whether or not the system in its entirety worked satisfactorily as planned also had to

be confirmed.

### **3.2. RP-HPLC with the contamination suppressing technique**

The contamination peaks were observed using pH buffer solutions of Tris, BES and HEPES at pH

7.5 without our suppression method. Since the blank peaks increased proportionally as the pH

buffer concentrations were increased and also subtly depended on the Ox concentration (as

1 shown in Figure 1), it was revealed that the main source of contamination was from the pH buffer  
2 reagents. The contamination from the Ox solution seemed to lie in  $10^{-8}$  M level in 0.0167 M Ox  
3 judging from the variation of peak heights as can be seen in Figure 1b. The typical chromatogram  
4 without the suppressing technique, i.e. without blocking reagents in each solution, is shown in  
5 Figure 2a and Figure 3a for the Tris and BES buffer, respectively. When the blocking reagents  
6 DHAB and pyrogallol were added to the stock solutions of the pH buffer and Ox, respectively,  
7 the blank peaks drastically decreased (Figure 2b and Figure 3b). When samples containing  $\text{Al}^{3+}$   
8 ions of  $1 \times 10^{-7}$  M were added to the pre-column mixture, the added  $\text{Al}^{3+}$  ions were reliably  
9 recovered, as was apparent in the increase of the peak height (Figure 2c and Figure 3c). It is clear  
10 that our contaminant suppression method was working as planned.

11 The typical content distribution of contaminant  $\text{Al}^{3+}$  is depicted in Figure 4 for the Tris buffer.  
12 The contribution of the contaminant Al to the blank peak broke down to 91 % and 3.15 % from  
13 the Tris and Ox stock solutions, respectively. It is noted that the  $\text{Al}^{3+}$  contamination even in high  
14 concentrations of pH buffer (1.5 M) and Ox solution (0.0167 M) was effectively suppressed.  
15 Such high concentrations of the reagents were practical requirements for taking measurements of  
16 real samples with pH-buffering and strong chelating ability, like biological samples. In fact, high  
17 concentrations of Ox (0.01 M) and the BES pH buffer (1.7 M) were set as the protocol for  $\text{Al}^{3+}$   
18 determination in serum and urine samples by the HPLC method using Ox [8].

19 It was necessary to experimentally confirm whether all the contaminant Al in the pH buffer  
20 stock solution was blocked at equilibrium, and that the ligand-exchange reaction  $(\text{Al}(\text{DHAB})_2 +$

1  $3\text{Ox} \rightarrow \text{Al}-(\text{Ox})_3 + 2\text{DHAB}$ ) did not occur at the pre-column derivatization step. This is necessary  
2 since, in contrast to the Ox-Py system in the Ox stock solution (*vide supra*), the blocking of  
3 contaminant  $\text{Al}^{3+}$  in the pH buffer solution was not able to be theoretically proved. Additional  
4 contamination  $\text{Al}^{3+}$  was intentionally mixed with the BES buffer stock solutions in order to  
5 simulate contaminated solutions. The time courses of the observed peak height when the blocking  
6 agent, DHAB, and additional contaminant Al were added to the stock solutions, are shown in  
7 Figure 5. The blank peak using the BES buffer stock solution including DHAB decreased for 5  
8 hours and reached equilibrium with the blank peak stably retained at low  $\text{ng ml}^{-1}$  levels. When  $1 \times 10^{-7} \text{ M Al}^{3+}$   
9 was mixed in the pH buffer stock solution together with DHAB, the blank peak  
10 also settled down in 10 hours at the same level as the solution without additional  $\text{Al}^{3+}$ . This  
11 suggests that all the contaminant  $\text{Al}^{3+}$  in pH buffer solution was successfully blocked as planned.  
12 This was supported by the fact that the peak heights of blanks using the different pH buffer  
13 reagents, Tris, BES and HEPES, were suppressed to almost the same blank value (1.6-2.2  $\text{ng ml}^{-1}$ ).  
14 Furthermore, the fact that the blank peak heights were the same value with and without  
15 additional contamination indicated that there was no exudation of  $\text{Al}^{3+}$  existing as DHAB  
16 complexes (which form the  $\text{Al}-(\text{Ox})_3$  complex through the ligand-exchange reaction). If the  
17 blocked  $\text{Al}^{3+}$  ions as DHAB complexes had dissociated to form Ox complexes, different peak  
18 heights with and without additional  $\text{Al}^{3+}$  should have been observed because larger contaminant  
19  $\text{Al}^{3+}$  content provides more dissociation of  $\text{Al}^{3+}$  to form the Ox complexes. The time course in  
20 Figure 5 also indicates that the suppression was quite stable after 10 hours.

There is an irremovable blank peak corresponding to 1.6-2.2 ng ml<sup>-1</sup> even when our suppression method is employed. The concentration of the blank is comparable to the blank value not to depend on the pH buffer and Ox concentrations, which is estimated from Figure 1. The peak height was proportional to the pH buffer concentration, as shown in Figure 1a. The intercept of the linear correlation corresponded to 2.5 ng ml<sup>-1</sup>. While no proportional correlation was obtained for contaminant Al in the Ox solution, it was revealed that the range of contamination level was in the range of sub-ng ml<sup>-1</sup> levels. Judging from the observation that the removal of contaminant Al<sup>3+</sup> from Ox solution was 0.80 ng ml<sup>-1</sup> ( $3 \times 10^{-8}$  M) in a 0.0167 M Ox solution (Figure 4), the blank was estimated to be around 1.7 ng ml<sup>-1</sup> when the contaminant was completely suppressed. This value is consistent with the observed blank peak height of 1.6-2.2 ng ml<sup>-1</sup>.

### 3.3. Performance of the contaminant suppressing technique

The detection limits (DL) were determined based on  $3\sigma$  of the blank peak for each pH buffer solution. The results are summarized in Table 1. The calibration curve was linear in the range of  $5 \times 10^{-8} - 1 \times 10^{-6}$  M for each set, and the correlation coefficients were over 0.999. The same slope values of the calibration curves were observed with or without blocking reagents within an error of  $\pm 4\%$ . This indicates that all the Al<sup>3+</sup> in samples was successfully recovered when our technique was employed, and that the complexation reaction with Ox in the mixed sample solution was significantly faster than that with blocking reagents (requirement 2,  $k_L \gg k_B$ ). If the present

1 system was unable to satisfy requirement 2, different slope values would have been observed due  
2 to the formation of the blocking agent complexes with  $\text{Al}^{3+}$  in the sample at the precolumn  
3 process. The fact that the DLs without the suppression technique ( $3\text{-}5\text{ ng ml}^{-1}$ ) were poorer than  
4 those reported previously ( $1\text{-}1.7\text{ ng ml}^{-1}$ ) [3,8], is considered to be attributable to our decision not  
5 to use additional purification steps for the pH buffer and Ox reagents in our study (previous  
6 studies used commercially available detection kits, in which the reagents are further purified).  
7 The DLs with our suppression technique were improved by a factor of 5.7-13.3, which were  
8 almost in agreement with the degree of suppression (with a maximum factor of 16.8 using Tris  
9 buffer, Fig. 4). The inter-day RSD of the blank peak was also improved from about 8 % to around  
10 4 % in our method (Table 1). This improvement of the RSD is likely to be due to the complete  
11 suppression of contamination from the reagents. The combination of the decrease and the  
12 stabilization of the blank peak provided improved sensitivity. Another advantage of our method is  
13 that low DLs were reliably obtained without the further purification of reagents and without fear  
14 of further contamination from outside after the preparation of the stock solutions. Any additional  
15 contamination  $\text{Al}^{3+}$  continuously forms complexes with the blocking agents.

16 The determination of Al in real samples, river waters (JAC0031 and JAC0032), was carried out  
17 as an example of an application of this system. The determined values of  $12.9$  and  $60.5\text{ }\mu\text{g L}^{-1}$  in  
18 our system were in good agreement with the certified values of  $13.4\pm0.7$  and  $61\pm2\text{ }\mu\text{g L}^{-1}$  in the  
19 presence of various other metal ions. This suggested that the proposed method was robust against  
20 other coexistent ions.

1 Although our contamination suppression technique successfully worked, problems still remain.

2 First, multiple injections resulted in a broadened peak of  $\text{Al}(\text{Ox})_3$  (150-300 injections). This is  
3 most likely due to the absorption of DHAB in the packing on the column top (the violet color of  
4 DHAB was observed on the packing). The removal of the colored packing and the repacking  
5 restored the performance. The employment of a guard column could possibly make this less of a

6 problem. Another problem is the oxidation of pyrogallol, which, left for a week or more, results  
7 in a deep brown coloration. It should be noted, however, that the performance of this technique  
8 was not affected. An additional problem is that a blank peak of  $\text{sub ng ml}^{-1}$  still remained. The  
9 source of the remaining contamination was thought to have been the instruments or surroundings  
10 since contamination from the reagents is suppressed for the most part. Because no large  
11 fluctuation of the remaining blank peak was observed (Table 1), it is unlikely that the blank is  
12 from the surroundings. An increase in the blank peak height was also observed when the injected  
13 sample stayed in the sample loop made by stainless steel for several minutes (data not shown).  
14 This implied the possibility of the contamination from the HPLC instruments themselves.

15 Although we employed an injector and tubes of polyether ether ketone (PEEK) resin to prevent  
16 contamination from stainless steel, no change in the residual peak was observed. This suggested  
17 the contamination came from not tubes but another part of instrument, such as, perhaps, a stator  
18 face of the injector made by alumina. The source of the contamination needs to be revealed in  
19 order to obtain a lower detection limit hereafter.

#### 1    **4. Conclusion**

2    We successfully developed a contamination suppressing technique in RP-HPLC by integrating  
3    kinetics and thermodynamics at pre-column processes with a simple procedure, i.e. only the  
4    addition of blocking agents to the solutions employed. This technique provides lower detection  
5    limits without the use of a clean room, which are given by the suppressed and stable blank peak.  
6    In this and our previous study [1], it is demonstrated that the principle of this technique is useful  
7    for trace level detection, and is applicable to various separation methods if one can find  
8    appropriate reaction system. This technique would be an option in the measurement when there is  
9    a possibility of much contamination.

10

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## Legends for Figures

**Figure 1.** Dependence of the blank peak height on the concentration of pH buffer and 8-quinolinol. a)  $C_{\text{Ox}} = 0.0167 \text{ M}$ , b)  $C_{\text{Tris}} = 0.667 \text{ M}$ .

**Figure 2.** Typical chromatogram of  $\text{Al}(\text{Ox})_3$  complex with Tris-HCl buffer using contamination suppressing technique. Sample,  $C_{\text{Ox}} = 0.0167 \text{ M}$ ,  $C_{\text{Tris}} = 1.5 \text{ M}$  (pH 7.5). a) blank, b) addition of DHAB and pyrogallol to pH buffer and Ox solution,  $C_{\text{DHAB}} = 3.3 \times 10^{-6} \text{ M}$ ,  $C_{\text{Py}} = 8.3 \times 10^{-7} \text{ M}$ , c) b) +  $1 \times 10^{-7} \text{ M Al}^{3+}$ .

**Figure 3.** Typical chromatogram of  $\text{Al}(\text{Ox})_3$  complex with BES buffer using contamination suppressing technique. Sample,  $C_{\text{Ox}} = 0.0167 \text{ M}$ ,  $C_{\text{BES}} = 1.5 \text{ M}$  (pH 7.5). a) blank, b) addition of DHAB and pyrogallol to the pH buffer and Ox solution,  $C_{\text{DHAB}} = 3.3 \times 10^{-6} \text{ M}$ ,  $C_{\text{Py}} = 8.3 \times 10^{-7} \text{ M}$ , c) b) +  $1 \times 10^{-7} \text{ M Al}^{3+}$ .

**Figure 4.** Content distribution of the contaminant  $\text{Al}^{3+}$  in HPLC. Experimental conditions are the same as in Figure 2.

**Figure 5.** Time course of the suppression process of contaminant  $\text{Al}^{3+}$  with DHAB in the pH buffer solution. Open triangle,  $[\text{DHAB}] = 1.0 \times 10^{-5} \text{ M}$  in 2.25 M BES stock solution,  $[\text{Ox}] = 1.0$

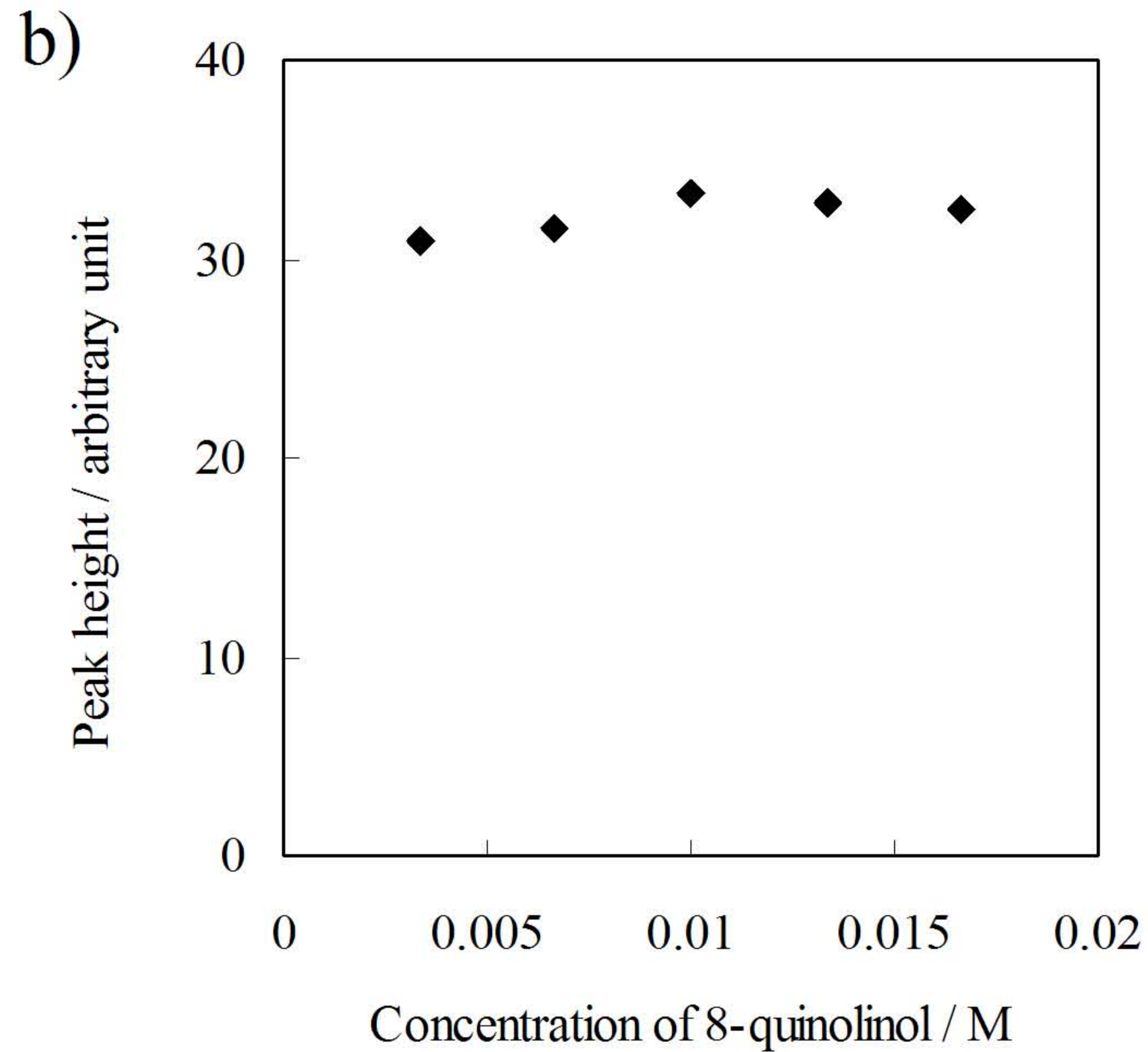
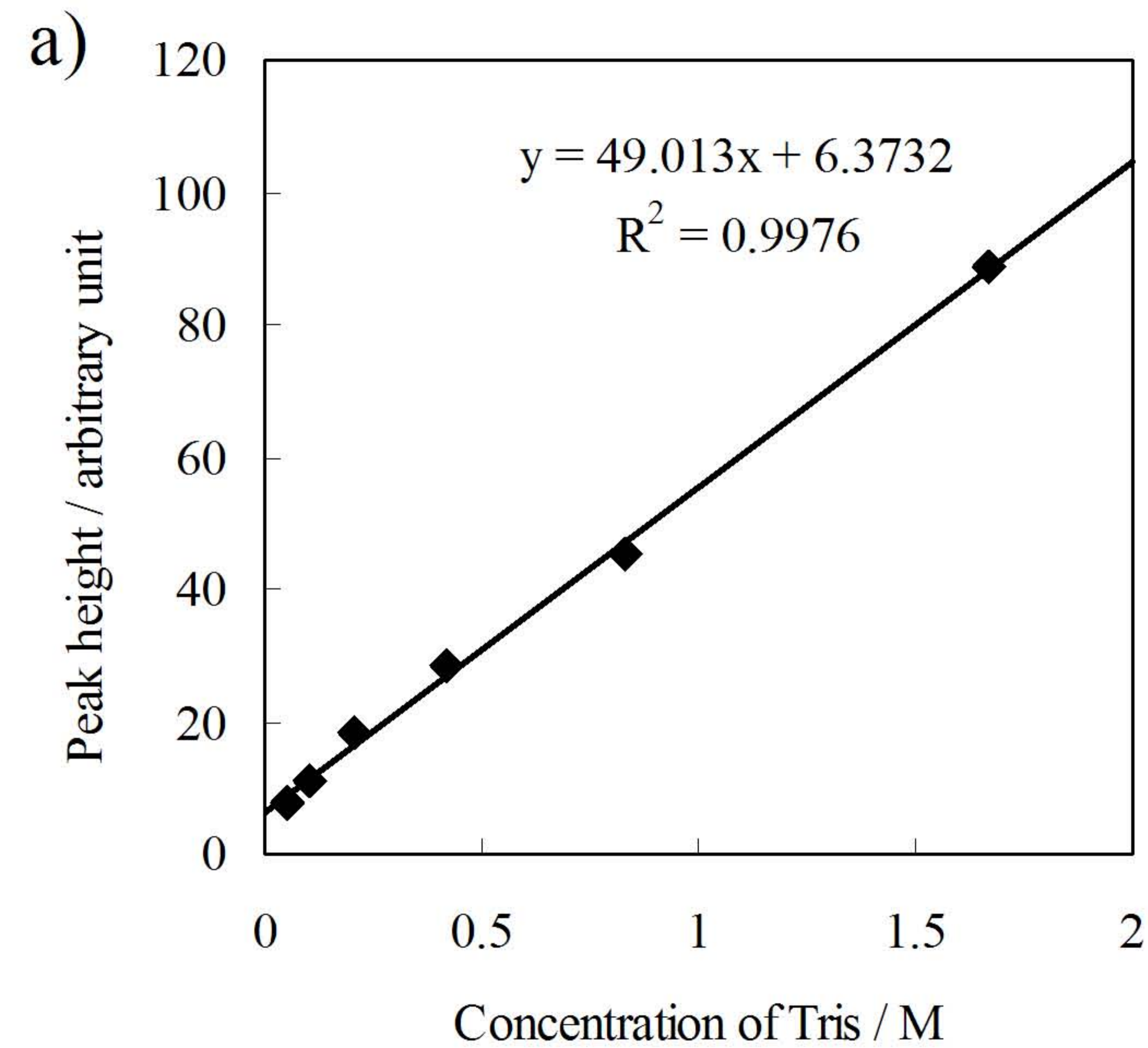
1  $\times 10^{-3}$  M, [BES] = 1.5 M, [DHAB] =  $6.6 \times 10^{-6}$  M in a sample solution. The closed circle,  $1.0 \times$   
2  $10^{-7}$  M of  $\text{Al}^{3+}$  was added to the stock pH buffer solution. The other conditions are the same as  
3 those of the closed circle.

4

5 **Table 1.** Comparison of detection limits with/without the contaminant suppression technique

pH buffer	Detection limit <sup>a</sup> / mol dm <sup>-1</sup> (ng ml <sup>-1</sup> )	Correlation coefficient of calibration curve <sup>b</sup>	RSD of blank peak height (%) Inter-day <sup>b</sup>
<i>Without suppression reagents</i>			
Tris	$2.0 \times 10^{-7}$ (5.3)	0.9994	7.7
BES	$1.6 \times 10^{-7}$ (3.8)	0.9997	7.0
HEPES	$1.2 \times 10^{-7}$ (3.2)	0.9994	8.6
<i>With suppression reagents</i>			
Tris	$1.5 \times 10^{-8}$ (0.41)	0.9999	1.7
BES	$2.2 \times 10^{-8}$ (0.59)	0.9998	4.0
HEPES	$2.1 \times 10^{-8}$ (0.58)	0.9995	4.5

6 <sup>a</sup> Detection limits were determined based on  $3\sigma$  of the blank peak heights ( $n = 5$ ). <sup>b</sup>  $n = 5$ .

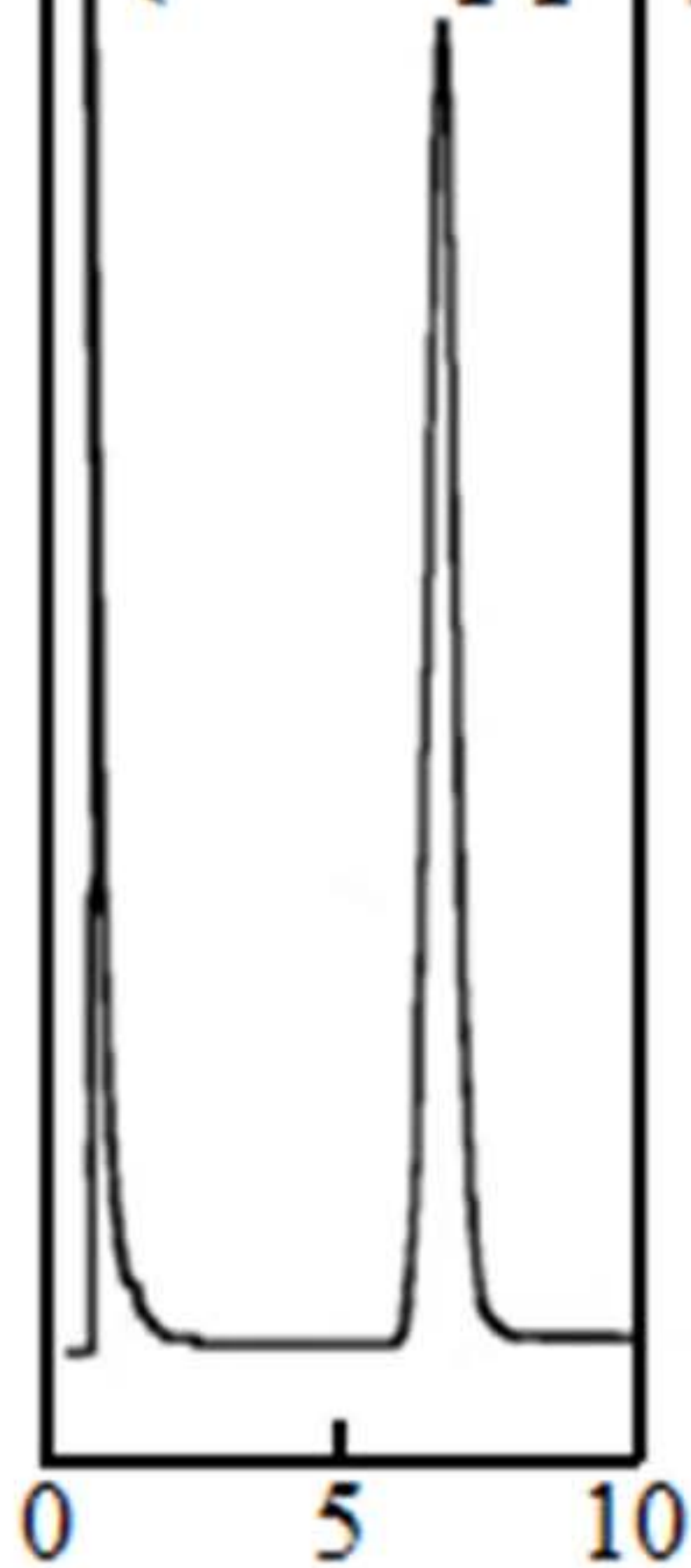


a)

Blank

 $\times 1/2$  $\text{Al}^{3+}$ 

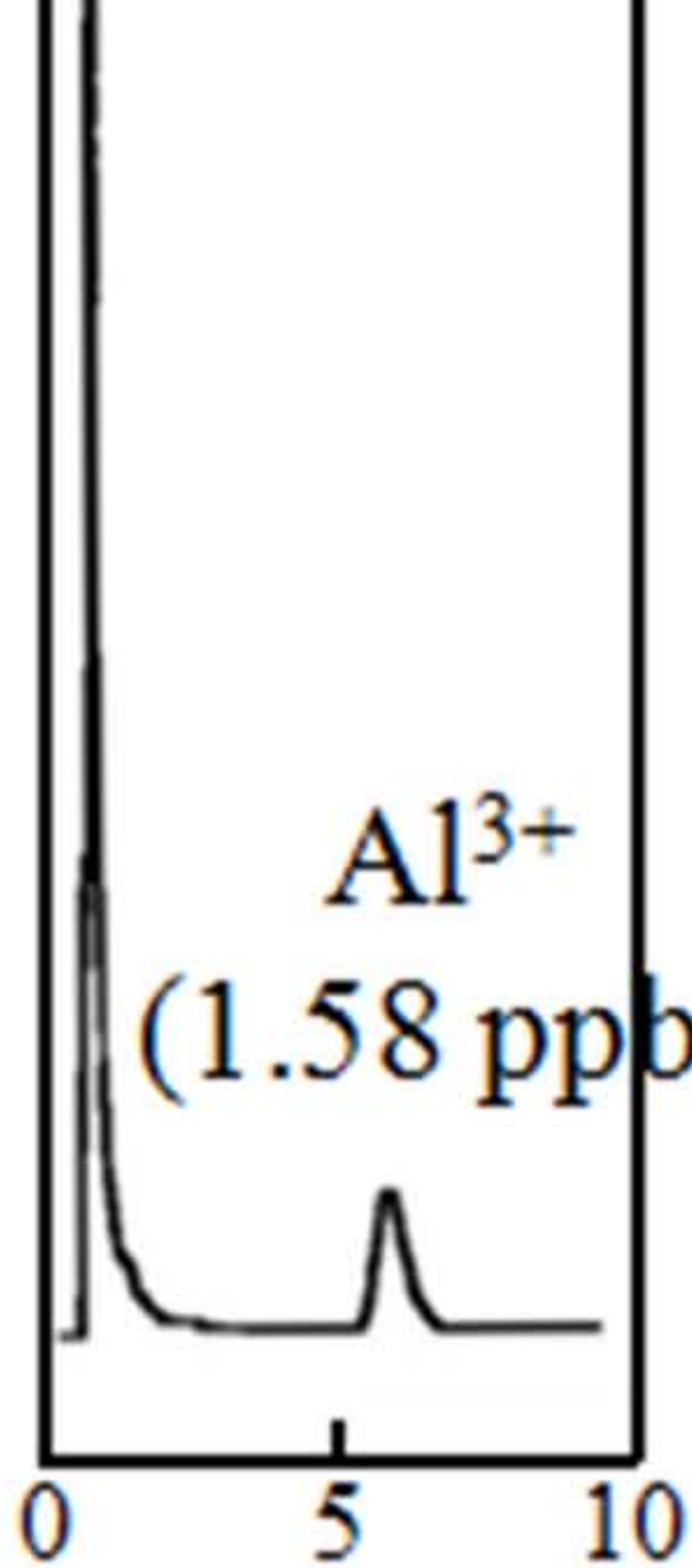
(26.6 ppb)



b)

Blank  
+  
Ox + Py $\text{Al}^{3+}$ 

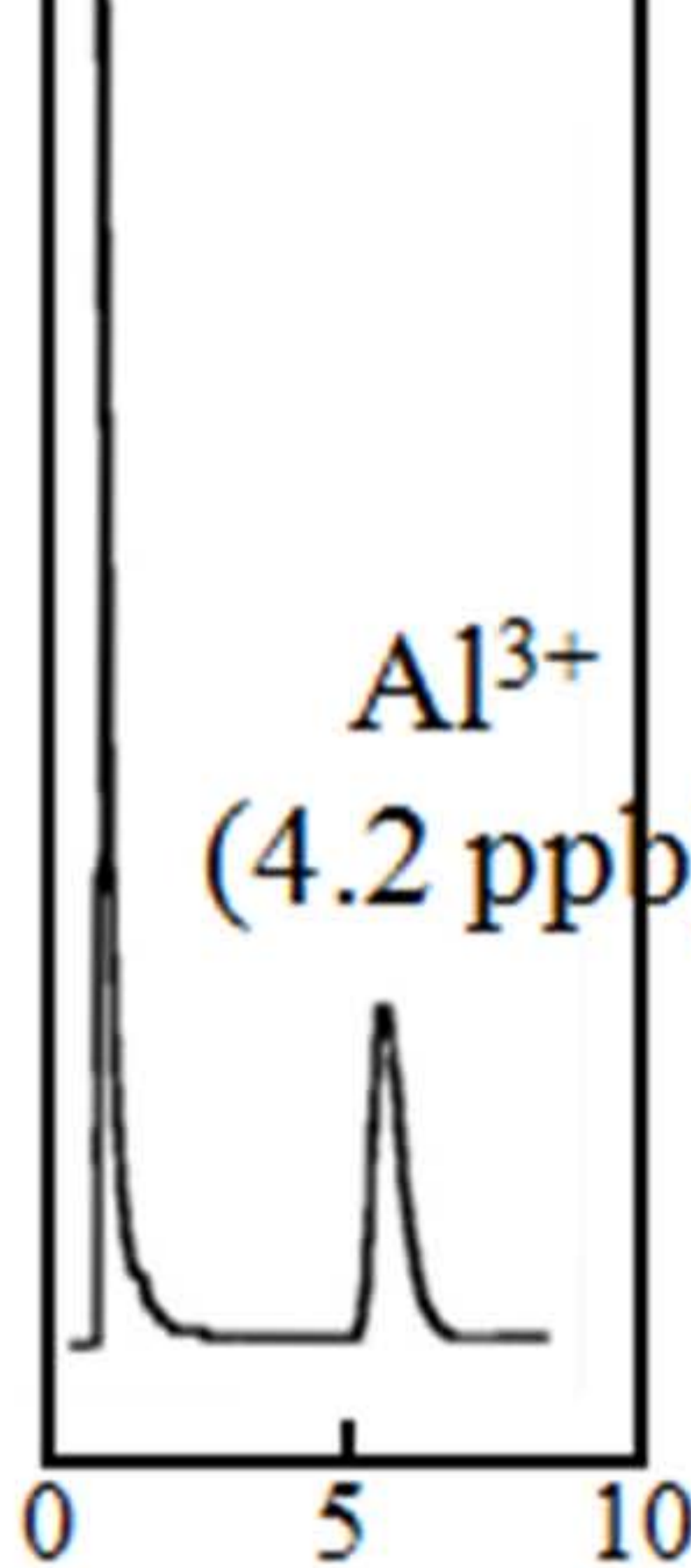
(1.58 ppb)



c)

Blank  
+  
Ox + Py  
+  
 $1 \times 10^{-7} \text{Al}^{3+}$  $\text{Al}^{3+}$ 

(4.2 ppb)



a)

Blank

 $\text{Al}^{3+}$   
(10.4 ppb)

0 5 10

b)

Blank  
+  
Ox + Py $\text{Al}^{3+}$   
(2.2 ppb)

0 5 10

c)

Blank  
+  
Ox + Py  
+  
 $1 \times 10^{-7} \text{Al}^{3+}$  $\text{Al}^{3+}$   
(4.9 ppb)

0 5 10

