< Original Paper> J. Flow Injection Anal., Vol. 32, No. 1 (2015) 5−8

# Application of Air Segmented Amplitude Modulated Multiplexed Flow Analysis with Software-Based Phase Recognition to the Determination of Ammonium Ion in Water Samples

Koji Inui[1], Haruka Yoshida[1], Masaki Takeuchi[1,2], and Hideji Tanaka[1,2,]*

[1] Faculty of Pharmaceutical Sciences, Tokushima University, Shomachi 1-78-1, Tokushima 770-8505, Japan
[2] Institute of Biomedical Sciences, Tokushima University, Shomachi 1-78-1, Tokushima 770-8505, Japan

## Abstract

Air segmented amplitude modulated multiplexed flow analysis (air segmented AMMFA) has been applied to the simultaneous determination of ammonium ions in water samples by an Indophenol Blue spectrophotometry. The flow rates of sample solutions to be merged are periodically varied at different frequencies. The merged solution, comprised of the sample solutions, coloring reagent solutions and water (diluent), is segmented by air bubbles so as to prevent the axial dispersion and thus to enhance the sensitivity. Both liquid and air segments are introduced to the flow cell of a UV/Vis detector and analytical signals at 660 nm are obtained. The signals corresponding to the air phase are removed by an in-house software. Thus deaerated signals are smoothed by a moving average computation and then analyzed by fast Fourier transform. The concentration of ammonium ion in each sample is determined from the amplitude of the corresponding wave component. Through the introductions of the air segmentation and the software-based phase recognition approaches, the sensitivity is increased maximally by a factor of 5.8 and lower detection limit (< 0.11 mg dm$^{-3}$ as N) is obtained compared with the previous non-segmented AMMFA. The proposed method has successfully been applied to the simultaneous determination of ammonium ions in real water samples.

**Keywords** Amplitude modulated multiplexed flow analysis, ammonium ion, Indophenol Blue, air segmentation, software-based phase recognition, fast Fourier transform

## 1. Introduction

Amplitude modulated multiplexed flow analysis (AMMFA) developed in our laboratory [1] is a sophisticated variation of continuous flow analysis for simultaneous determination. The pieces of information on the samples to be merged are modulated by periodically varying their flow rates at different frequencies. Downstream, analytical signals for the merged solution are obtained and are analyzed by fast Fourier transform (FFT) [1,2]. The analytes in the samples are determined based on the amplitudes of corresponding wave components of the signals. AMMFA has been applied to the determinations of ferrous ions [1] and chloride ions [2]. The introduction of air segmentation technique, which had been originated by Skeggs [3,4] and extensively studied since then [5-12], to AMMFA could improve the sensitivity because the axial dispersion was limited within each liquid segment separated by air bubbles [13]. We applied the air-segmented AMMFA to the simultaneous determination of nitrite and nitrate ions in a single sample [14]. In this approach, air bubbles had to be removed just before the detection because air signals would interfere with the FFT analysis. A piece of porous PTFE tubing was used for the deaeration. However, considerable amplitude damping still occurred after the deaeraton. In the previous study [15], we adopted a software-based approach for the removal of air signals instead of the physical deaeration. Air bubbles as well as liquid segments were introduced to the flow cell of a detector. Analytical signals corresponding to the air phase were removed by a signal processing with an in-house program. The method was applied to the determination of phosphate ion in water samples. The sensitivity was increased maximally by a factor of 1.66 compared with the previous non-segmented AMMFA.

In the present study, we further applied the air segmented AMMFA to the determination of ammonium ion in water samples by an Indophenol Blue method [16,17] in order to evaluate the versatility of the method. Ammonium-nitrogen is an important index of water pollution caused by domestic

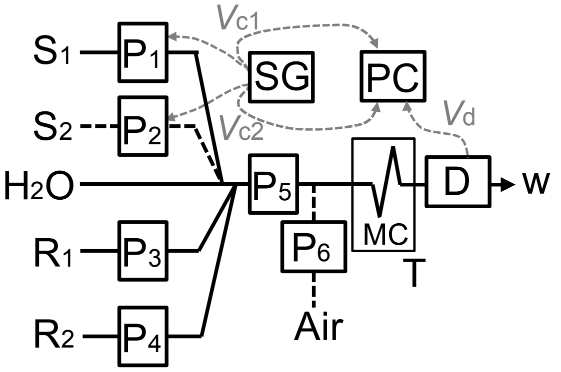

Fig. 1 Schematic diagram of flow system. $S_1$ and $S_2$, sample solutions; $R_1$ and $R_2$, reagent solutions; $H_2O$, water (diluent); w, waste; $P_1$ − $P_6$, peristaltic pump; MC mixing coil; T, thermostat bath; D, UV/Vis detector; SG, signal generator; PC, laptop computer with a card type A/D-D/A converter; $V_{c1}$ and $V_{c2}$, controller output voltages; $V_d$, detector output voltage.

---

*To whom correspondence should be addressed.
E-mail: h.tanaka@tokushima-u.ac.jp

sewage, factory waste, effluent from fertilized soil and so on. Although the color reaction is susceptible to experimental conditions such as reaction time, pH and temperature, flow-based approaches such as flow injection analysis [18-23] and sequential injection analysis [24,25] can control such conditions precisely. The present air segmented AMMFA gives maximally 5.8 times higher sensitivity and lower detection limit (< 0.11 mg dm$^{-3}$ as N) than those obtained by the previous non-segmented AMMFA. The method has been applied to the simultaneous determinations of ammonium ions in real water samples.

## 2. Experimental

### 2.1. Flow system

Figure 1 shows the schematic diagram of the flow system configured in the present study. A four channels system (solid lines) was used for the optimization of fundamental experimental conditions (*i.e.*, reaction temperature, mixing coil length, flow rates and concentrations of reagents). A six channels system (solid and broken lines) was used for the simultaneous determination of ammonium ions in two samples. Five peristaltic pumps ($P_1 - P_3$; Rainin Dynamax RP-1, USA and $P_4$ and $P_5$; Gilson Miniplus 3, France) were used for delivering solutions. One pump ($P_6$; the same model as $P_4$ and $P_5$) was used for feeding air. The pump tubes used were Pharmed® tubing (0.80 mm i.d. for $P_5$ and 0.51 mm i.d. for the others). The flow rates of sample solutions ($S_1$ and $S_2$) delivered by $P_1$ and $P_2$ were respectively varied in the range from 0 to 0.25 cm$^3$ min$^{-1}$ in response to the sinusoidal controller output voltages ($V_{c1}$ and $V_{c2}$; both amplitudes are 0 – 5 V (peak to peak)) with different frequencies (typically 0.05 and 0.10 Hz for $V_{c1}$ and $V_{c2}$, respectively). The voltages were generated from a signal generator (SG; NF Corp. WF1974, Japan). Reagents solutions ($R_1$ and $R_2$; described in detail in Section 2.3) were delivered by $P_3$ and $P_4$, respectively. Their flow rates were held constant at 0.5 and 0.3 cm$^3$ min$^{-1}$, respectively. The total flow rate was also held constant at 1.4 cm$^3$ min$^{-1}$ with $P_5$. The merged solution was segmented by air bubbles introduced from the sixth channel at the flow rate of 0.3 cm$^3$ min$^{-1}$. Both liquid and air segments passed through a mixing coil (MC; 0.5 mm i.d., 5 m length) immersed in a thermostat bath (T; 70℃). The temperature of the bath was controlled by an AS ONE CA061 heater, a Shinko PT100 resistance thermometer and a Shikoku-Riken ACS-13A PID controller. Thus colored solution was introduced to a hand-made quartz flow cell (optical path length: 1 mm) set in a UV/Vis detector (D; Shimadzu SPD-6AV, Japan) without any physical deaeration. The analytical wavelength was 660 nm. Detector output voltages ($V_d$, 0.8 V/AU) as well as $V_{c1}$ and $V_{c2}$ were quantized by an A/D-D/A converter (Measurement Computing, PC-CARD-DAS16/12-AO, USA) and acquired in a computer (PC; IBM ThinkPad R51e 1843-BLJ, USA) as a Microsoft Excel format. An in house program written in Visual BASIC was used to acquire, analyze and display the data automatically.

### 2.2. Reagents and samples

Reagents of analytical reagent grade purchased from Kanto Chemicals (Tokyo, Japan) or Nacalai Tesque (Kyoto, Japan) were used without further purification. Zartorius Arium 611DI grade deionized water was used throughout. Ammonium standard solutions were prepared by dissolving appropriate amounts of ammonium chloride in water. Real samples were collected from the Naka Pond, the Iio River, the Hukuroi Irrigation Canal and the Moat of Shozui Castle in Tokushima Prefecture, Japan. The samples were filtered through disposable disk filters with 0.45 μm pore size (Kanto Chemical Co., Tokyo, Japan), and were stored in polypropylene bottles at 4ºC.

### 2.3. Determination of ammonium ion

Ammonium ion was determined by an Indophenol Blue method. Salicylic acid instead of phenol was used because of instability and toxicity of phenol [17,18]. Monochloramine produced from ammonium ion and hypochlorous acid reacted with salicylic acid to generate Indophenol Blue. The absorbance was measured at 660 nm. One reagent, $R_1$ (see Fig. 1), was prepared by mixing an aqueous solution containing sodium salicylate (300 g dm$^{-3}$) and sodium tartrate (38 g dm$^{-3}$) with that of sodium pentacyanonitrosylferrate(III) dihydrate (0.75 g dm$^{-3}$) at the volume ratio of 1:1 just before the measurement. The other reagent, $R_2$, was prepared by mixing an aqueous solution of 1 mol dm$^{-3}$ sodium hydroxide with that of 5.2% sodium hypochlorite at the volume ratio of 9 : 1.

### 2.4. Signal processing

Typical software parameters are listed in Table 1. The principles of softwase-based phase recognition [15] and of FFT for AMMFA [1] were described in detail previously. Two kinds of threshold values (*i.e.*, the values for the $V_d$ slope ($dV_d/dt$) and the $V_d$ deviation from the latest liquid signal ($\Delta V_d$)) are set in order to distinguish sharp air signals from rather flat liquid signals. The values are determined through the preliminary experiments. If newly acquired $V_d$ exceeds either of the threshold values, it is regarded as air signal and discarded. The latest liquid signal is held in this case. The signals are further smoothed by a moving average using 31 data. Eight data of resulting $V_d$ at equal intervals in an FFT window are used for FFT analysis. The least common multiple of the periods of $V_{c1}$ and $V_{c2}$ are set as the FFT window. The concentrations of ammonium ions in the samples are obtained from the amplitudes of respective wave components. FFT analysis is carried out every 1.25 s by moving the window with time.

## 3. Result and Discussion

### 3.1. Optimization of analytical conditions for a coloring reaction

Table 1 Typical software parameter values

| Software parameters | Typical values |
|---|---|
| Sampling frequency / Hz | 12.8 |
| Threshold value of $dV_d/dt$ / V s$^{-1}$ | 1.2 |
| Threshold value of $\Delta V_d$ / V | 0.14 |
| Number of data for moving average | 31 |
| Number of data for FFT analysis | 8 |
| Interval of FFT analysis / s | 1.25 |

Analytical conditions listed in the leftmost column in Table

Table 2 Optimization of analytical conditions

| Parameters | Studied ranges | Optimal values |
|---|---|---|
| Temperature of thermostat bath / °C | 30 − 80 | 70 |
| Mixing coil length / m | 1 − 7 | 5 |
| Flow rate of $P_3$ / $cm^3$ $min^{-1}$ | 0.1 − 0.8 | 0.5 |
| Flow rate of $P_4$ / $cm^3$ $min^{-1}$ | 0.1 − 0.8 | 0.3 |
| Concentration of sodium salicylate /g $dm^{-3}$ | 20 − 400 | 300 |
| Concentration of sodium pentacyanonitrosylferrate (III) dehydrate /g $dm^{-3}$ | 0.15 − 3.0 | 0.75 |
| Concentration of sodium hydroxide / mol $dm^{-3}$ | 0.05 − 20 | 1 |
| Concentration of sodium hypochlorite*, % | 2.1 – 0.05 | 0.52 |

* Prepared from sodium hyochlorite solution (available chlorine: approximately 5%)

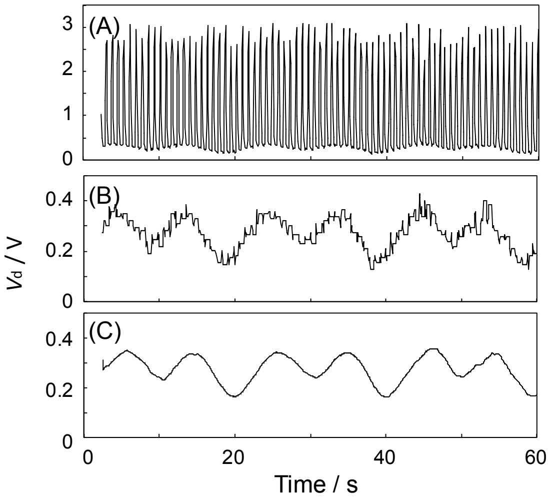

Fig. 2 Flow signals. $S_1$ and $S_2$ were 1.0 mg $dm^{-3}$ ammonium solutions. A, raw $V_d$; B, software-based deaerated $V_d$; C, software-based deaerated and moving average smoothed $V_d$.

2 were investigated successively by one-factor-at-a-time approach from that in the upper row to in the lower row using 2.0 mg $dm^{-3}$ as N (0.014 mmol $dm^{-3}$) ammonium solutions. The investigated ranges and the optimized conditions are listed in the middle and the rightmost columns.

Typical analytical signals are shown in Figs. 2A-C, where both $S_1$ and $S_2$ are 1.0 mg $dm^{-3}$ as N (0.071 mmol $dm^{-3}$) ammonium solutions. Figure 2A shows the raw $V_d$ signals. Steep upward peaks correspond to air phase. Bottom envelope of $V_d$ trace is, therefore, corresponds to liquid phase. Figure 2B shows the $V_d$ after the removal of air signals by the signal processing. The threshold values for the recognition of air signals were 1.2 V $s^{-1}$ and 0.14 V for $dV_d/dt$ and $\Delta V_d$, respectively. The $V_d$ is of stair-like trace because the latest liquid $V_d$ instead of newly acquired $V_d$ was held when the latter was regarded as air signal, as described in Section 2.4. Figure 2C shows the software-based deaerated and moving average smoothed $V_d$, where successive 31 data in Fig. 2B are averaged. The results show that the developed software can successfully recognize and remove air signals.

### 3.2. Analytical performance

Calibration curves were constructed from five standard solutions containing 0 − 2.0 mg $dm^{-3}$ (as N) ammonium ions, by taking the dynamic range of the official FIA method with the same color reaction (*i.e.*, 0.05 − 10 mg $dm^{-3}$ as N) [17] into consideration. Analytical performance of the present method (air segmented AMMFA with no physical deaeration) was compared with that of the previous method (unsegmented AMMFA) [1,2], as shown in Table 3. In this experiment, the control periods of $V_{c1}$ (for $S_1$) and $V_{c2}$ (for $S_2$) were 20 s and 10 s, respectively; the length of FFT window was automatically set at 20 s (the least multiple of the control periods). The amplitudes of the fundamental and the second harmonic wave components are, therefore, correspond to the concentrations of ammonium ions in $S_1$ and $S_2$, respectively. The present method gave higher linearity than the previous method. The linearity of the previous method for $S_2$ was poor ($r^2$ = 0.7843) because $V_{c1}$ period (10 s) for $S_2$ was too short to get reliable data under unsegmented condition. That is, the shorter the control period is, the lower the amplitude becomes [2], because steeper concentration gradient in the conduit resulted in greater dispersion. In addition, it is reasonable to consider that a detecor having a time constant (0.05 s in our instrument) cannot follow rapid concentration change completely when the control period became shorter. The amplitude obtained by air

Table 3 Calibration curves for the simultaneous determination of ammonium ion in two samples

| Sample | Air-segmented AMMFA with software-based phase recognition (present method) | | | | AMMFA (previous method) | | | |
|---|---|---|---|---|---|---|---|---|
| | Slope | Intercept | $r^2$ | LOD / mg $dm^{-3}$ | Slope | Intercept | $r^2$ | LOD / mg $dm^{-3}$ |
| $S_1$ | 0.0789 | 0.0044 | 0.9993 | 0.101 | 0.0438 | 0.0086 | 0.9979 | 0.176 |
| $S_2$ | 0.0353 | 0.0109 | 0.9992 | 0.111 | 0.0061 | 0.0081 | 0.7843 | 1.998 |

Calibration curves are expressed as $A$ = slope × $C$ + intercept. $A$ is the amplitude of fundamental wave component of $V_d$. $C$ is the concentration of analyte in mg $dm^{-3}$. The flow rate of $S_1$ and $S_2$ were varied with the periods of 20 and 10 s, respectively.

Table 4 Determination of ammonium ion in real water samples by air-segmented AMMFA

| Channel | Set #1 | | Set #2 | |
|---|---|---|---|---|
| | Sample | $C_{NH_4^+}$ / mg dm$^{-3}$ as N | Sample | $C_{NH_4^+}$ / mg dm$^{-3}$ as N |
| $S_1$ | Naka Pond | 1.00 ± 0.04 | Hukuroi Irrigation Canal | 1.89 ± 0.03 |
| $S_2$ | Iio River | 1.02 ± 0.03 | Moat of Shozui Castle | 0.99 ± 0.03 |

segmented AMMFA was higher than that by unsegmented AMMFA, as expected. The introduction of air bubbles is also effective for the improvement of the limit of detection (LOD at 3.3$\sigma$). Some compromise is, however, needed between LOD and the multiple analysis nature of AMMFA, because multiple samples are simultaneously introduced to the flow system. Relative standard deviation for the repeating measurements ($n$ = 10) for $S_1$ and $S_2$ (both in 1.0 mg dm$^{-3}$) were 2.08% and 3.48%, respectively, in air segmented AMMFA.

### 3.3. Application to real water samples

The present air segmented AMMFA was applied to the simultaneous determination of ammonium ions in real samples. In the first set, the Naka Pond water and the Iio River water were introduced from the channels of $S_1$ and $S_2$, respectively. In the second set, the Hukuroi Irrigation Canal water and the Moat of Shozui Castle water were done so, respectively. The analytical values (mean ± s.d. ($n$ = 3)) for the water samples are listed in Table 4. The ions in the samples were also determined independently by a conventional continuous flow method with neither amplitude modulation nor air-segmentation [22], as a reference. Obtained values are 1.03 ± 0.02, 1.04 ± 0.04, 1.86 ± 0.02 and 1.00 ± 0.02 mg dm$^{-3}$, respectively. No significant differences were observed between the results obtained by the air segmented AMMFA and the conventional flow analysis at the 95% confidence level ($t$-test). It is concluded, therefore, air segmented AMMFA with software-based phase recognition is well applicable to the determination of ammonium ion in real water samples.

## 4. Conclusion

Air segmented amplitude modulated multiplexed flow analysis (air segmented AMMFA) with software-based phase recognition proposed in the previous study [15] has further been applied to the simultaneous determinations of ammonium ions in multiple samples. The present method can give higher sensitivity and lower LOD value compared with AMMFA with no air segmentation (our previous method [1,2]). Air segmented AMMFA has successfully been applied to the analysis of real water samples.

## 5. Acknowledgments

The present study is partly supported by the Grant-in-Aid for Scientific Research (C) (24550101) from the Japan Society for the Promotion of Sciences (JSPS).

## References

[1] H. Tanaka, T. Mima, M. Takeuchi, H. Iida, *Talanta*, **77**, 576 (2008).

[2] Y. Kurokawa, M. Takeuchi, H. Tanaka, *Anal. Sci.*, **26**, 791 (2010).

[3] L. T. Skeggs, *Am. J. Clin. Pathol.*, **28**, 311 (1957).

[4] L.T. Skeggs, *Anal. Chem.*, **38**, 31A (1966).

[5] L. Snyder, J. Levine, R. Stoy, A. Conetta, *Anal. Chem.*, **48**, 942 (1976).

[6] L. R. Snyder, H. J. Adler, *Anal. Chem.*, **48**, 1017 (1976).

[7] L. R. Snyder, H. J. Adler, *Anal. Chem.*, **48**, 1022 (1976).

[8] C. Pasquini, W. A. de Oloveira, *Anal. Chem.*, **57**, 2575 (1985).

[9] C. Pasquini, *Anal. Chem.* **58**, 2346 (1986).

[10] L. C. Tian, X. P. Sun, Y. Y. Xu, Z. L. Zhi, *Anal. Chim. Acta*, **238**, 183 (1990).

[11] Y. Hsieh, S. R. Crouch, *Anal. Chim. Acta*, **303**, 231 (1995).

[12] Z.-L. Zhi, *Trends Anal. Chem.*, **17**, 411 (1998).

[13] K. Inui, T. Ogusu, M. Takeuchi, H. Tanaka. *Anal. Sci.*, **27**, 305 (2011).

[14] H. Yoshida, K. Inui, M. Takeuchi, H. Tanaka. *Anal. Sci.*, 28, 523 (2012).

[15] T. Ogusu, K. Uchimoto, M. Takeuchi, H. Tanaka, *Talanta*, **118**, 123 (2014).

[16] JIS K 0102, “Testing Methods for Industrial Wastewater (in Japanese)”, **1994**, Japanese Industrial Standards Committee, Tokyo, Japan.

[17] JIS K 0170-1, “Testing Methods for Water Quality by Flow Analysis − Part 1 Ammonium Nitrogen (in Japanese)”, **2011**, Japanese Industrial Standards Committee, Tokyo, Japan.

[18] H. Muraki, K. Higuchi, M. Sasaki, T. Korenaga, K. Tôei, *Anal. Chim.. Acta*, **261**, 345 (1992).

[19] A. Cerda, M. T. Oms, R. Forteza, V. Cerda, *Anal. Chim. Acta*, **311**, 165 (1995).

[20] K. Higuchi, H. Nuraki, Y. Ogawa, *J. Flow Inject. Anal.*, **16**, 47 (2000).

[21] K. Fukui, S. Ohno, K. Higuchi, N. Teshima, T. Sakai, *Bunseki Kagaku*, **56**, 757 (2007).

[22] K. Higuchi, K. Takahashi, N. Teshima, T. Sakai, *Bunseki Kagaku*, **57**, 631 (2008).

[23] Y.Z.D. Yuan, Y.H.J. Ma, S. Feng, K. Lin, *Marine Chem.*, **162**, 114 (2014).

[24] J. F. van Staden, R. E. Taljaard, *Anal. Chim. Acta*, **344**, 281 (1997).

[25] C.M.C. Infante, J.C. Mashini, A.C.V. dos Santos, *Microchem. J.*, **98**, 97 (2011).

(Received May 19, 2015)
(Accepted June 15, 2015)