

Internal Standard-Amplitude Modulated Multiplexed Flow Analysis

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A new concept of flow analysis, internal standard-amplitude modulated multiplexed flow analysis, is proposed. A proof of concept was verified by applying it to the determination of ferrous ion (Fe^{2+}) by 1,10-phenanthroline (*o*-Phen) spectrophotometry. The flow rates of sample solutions containing Methylene Blue (MB) as an internal standard substance were sinusoidally varied at different frequencies. The solutions were merged with a color reagent (*o*-Phen) solution, while the total flow rate was held constant. Downstream, analytical signals were monitored at the maximum absorption wavelengths of Fe^{2+} -*o*-Phen complex and of MB (510 and 644 nm, respectively). The signals were respectively analyzed by fast Fourier transform. The concentrations of the analytes in respective samples were simultaneously determined from the amplitudes of the corresponding wave components. The precision, linearity of the calibration curve, limit of detection and robustness against deliberate fluctuation in flow rate were greatly improved by introducing the internal standard method. Good recoveries of around 100% were obtained for Fe^{2+} spiked into real water samples.

Keywords Amplitude modulated multiplexed flow analysis, amplitude modulation, internal standard method, Fourier transform, 1,10-phenanthroline, Fe(II) ion

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Introduction

Amplitude modulated multiplexed flow analysis (AMMFA), proposed by our group in 2008,¹ is a continuous flow analysis that is coupled with frequency analysis. Pieces of information on analytes in sample solutions are amplitude modulated by varying the flow rates at different frequencies. The sample solutions are merged with a reagent solution, and, downstream, analytical signals are monitored. The signals are analyzed in real time by fast Fourier transform (FFT). The concentrations of analytes in the samples are simultaneously determined from the amplitudes of corresponding wave components. We have applied the concept to the determinations of food dyes,¹ ferrous ion,¹ chloride ion,² nitrite ion,³ phosphate ion^{3,4} and ammonium ion⁵ in multiple samples, and to the simultaneous determinations of nitrite and nitrate ions⁶ in a sample.

AMMFA is less susceptible to baseline drift because the quantification is not based on the values of analytical signals, but on the amplitudes of analytical signals.⁷ However, as well as other flow analyses, the error will be included in the analytical values if unexpected fluctuation in the flow rate occurs. Reconstruction of the calibration curve at appropriate intervals is, therefore, necessary for a long-time measurement. This requirement would be a disadvantage because any measurement of the samples must be discontinued during the calibration.

The internal standard method can compensate for several types of both random and systematic errors if an internal standard substance is properly chosen and used.⁸ The method has extensively been used in gas chromatography, atomic spectrometry and so on. In addition to compensation for any fluctuation in sample introduction, it can correct any fluctuation in the flow rate in flow analysis, if the signals for the analyte and the internal standard are influenced in the same manner by the fluctuation. Gibb *et al.*,⁹ employed two internal standards in their flow injection-gas diffusion-ion chromatography. *sec*-Butylamine was added to the acceptor solution in order to monitor the stability and reproducibility of the chromatography, whilst cyclopropylamine was spiked to sample solutions to quantify NH_3 and methylamines using predetermined relative response factors. Gimenes *et al.*¹⁰ used $[\text{Fe}(\text{CN})_6]^{3-}$ as an internal standard in their system of flow injection-amperometry in order to compensate for variations in the analytical parameters, including the flow rate.

In the present study, the internal standard method is introduced to AMMFA so as to improve the robustness against any fluctuation in the sample flow rate. The determination of ferrous ion (Fe^{2+}) by 1,10-phenanthroline (*o*-Phen) spectrophotometry¹¹ is selected for verifying the proof of concept, because a stable colored complex is stoichiometrically produced. The spectrophotometry has been employed to evaluate new concepts of analysis. For example, Tsunoda *et al.*¹² showed the applicability of their glass optical waveguide to spectrophotometry by using *o*-Phen spectrophotometry. Olivera *et al.*¹³ proposed an asynchronous merging zone method

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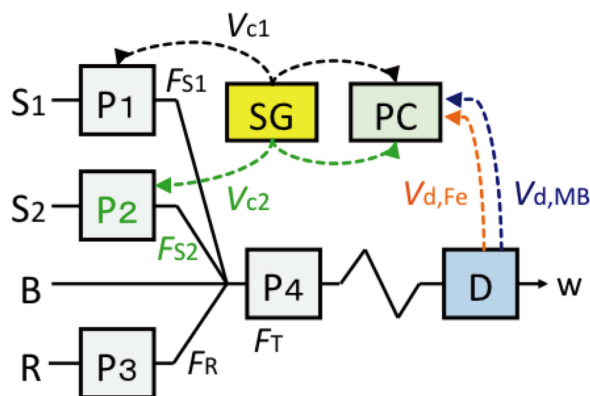


Fig. 1 Flow system of internal standard-amplitude modulated multiplexed flow analysis. S_1 and S_2 , Sample solutions including internal standard substance; R, reagent solution; B, diluent (buffer solution); P_1 – P_4 , peristaltic pumps; D, UV/Vis dual-wavelength spectrophotometer; SG, signal generator; PC, laptop computer with card type A/D-D/A converter; V_{c1} and V_{c2} , sinusoidal controller output voltages each having a different frequency; $V_{d,Fe}$ and $V_{d,MB}$, detector output voltages corresponding to the analyte (Fe^{2+}) and internal standard substance (MB), respectively. F_{S1} , F_{S2} , F_R and F_T are flow rates of S_1 , S_2 , R and merged solutions, respectively.

based on *o*-Phen spectrophotometry. It has been revealed that the precision, linearity of the calibration curve, limit of detection and the robustness against any deliberate fluctuation in the flow rate of AMMFA are greatly improved by introducing the internal standard method.

Experimental

Reagents and samples

Ammoniumiron(II)sulfatehexahydrate ($Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$) and Methylene Blue (MB) were purchased from Nacalai Tesque (Kyoto, Japan). 1,10-Phenanthroline monohydrate and hydroxylammonium chloride were purchased from Kanto Chemicals (Tokyo, Japan). These reagents were of analytical reagent grade and were used without further purification. Zartorius Arium 611 DI grade deionized water (resistivity, 18.2 M Ω cm) was used throughout.

A buffer solution (pH 4.6) was prepared by dissolving 4.8 cm³ of acetic acid (99.7%) and 4.45 g of sodium acetate trihydrate in water to make a final volume of 1 dm³. An iron(II) solution (1 mmol dm⁻³) was daily prepared by dissolving 0.3923 g of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ in the acetate buffer solution. The solution was diluted with the buffer solution to prepare Fe^{2+} standard solutions (0–0.1 mmol dm⁻³). The color reagent solution comprised 5.04 \times 10⁻⁴ mol dm⁻³ *o*-Phen and 4.80 \times 10⁻² mol dm⁻³ hydroxyammonium chloride, which were dissolved in the buffer solution.

Real water samples were collected from the Fukuroi Irrigation Canal, the moat of Shozui Castle, the Yoshino River, the Naka Pond and a hot spring in Tokushima Prefecture, Japan. The samples were each filtered through a disposable disk filter with a pore size of 0.45 μ m (Kanto Chemical Co. 96904-00, Tokyo, Japan). The filtrates were stored in clean polypropylene bottles in a refrigerator at 4°C.

Standard and sample solutions were, respectively, spiked with 0.01 mmol dm⁻³ MB as an internal standard substance.

Table 1 Typical software parameters

Principal software parameter		Typical value
Control signal V_{c1}	Amplitude/V	0–5 ^a
	Period/s	30
Control signal V_{c2}	Amplitude/V	0–5 ^a
	Period/s	20
Sampling frequency/Hz		8.533
Number of data for FFT computation		8
Interval of FFT computations/s		1.875

a. Peak to peak voltage.

Flow system

Figure 1 shows the flow system fabricated in the present study. Four peristaltic pumps (P_1 – P_4 ; Rainin Dynamax RP-1, USA) were used for delivering solutions. PharMed tubing with 0.51 mm i.d. was used for P_1 – P_4 as pump tubes. The flow rates (F_{S1} and F_{S2}) of sample solutions (S_1 and S_2) delivered using P_1 and P_2 , respectively, were varied in the range from 0 to 0.3 cm³ min⁻¹ in response to sinusoidal controller output voltages (V_{c1} and V_{c2}) with different frequencies. The voltages were generated from a signal generator (SG; NF Corp. WF1974, Japan). The color reagent solution (R) was delivered by P_3 at a flow rate of 0.3 cm³ min⁻¹. The total flow rate was held constant at 1 cm³ min⁻¹ with P_4 . The flow rate of the diluent (B, the acetate buffer solution) aspirated to the confluence point was, therefore, $F_T - F_{S1} - F_{S2} - F_R$. In the merged solution, Fe^{2+} in the sample solutions reacted with *o*-Phen supplied from the color reagent solution at a molar ratio of 1:3 to form an orange-red complex ($\lambda_{max} = 510$ nm). Thus colored solution was introduced to a UV/Vis dual-wavelength detector (D; GL Sciences UV702, Japan). The analytical wavelengths were 510 and 664 nm for measurements of the analyte (*i.e.*, Fe^{2+}) and the internal standard substance (*i.e.*, MB), respectively. The corresponding detector output signals ($V_{d,Fe}$ and $V_{d,MB}$), 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). The resulting digital data were acquired in a laptop computer (PC; Toshiba Dynabook Satellite 1850 SA120C/4, Japan) in Microsoft Excel format.

In some experiments, cosine waves or triangular waves, the periods of which were different from those of V_{c1} and V_{c2} , were superimposed to V_{c1} and V_{c2} so as to verify the robustness of the present system against any fluctuation in F_{S1} and F_{S2} .

Software

An in-house program written in Excel VBA was used for acquiring signals, analyzing them and displaying the results automatically. The FFT algorithm was referred to a book authored by Nakamura.¹⁴ Table 1 shows the typical analytical parameters. The control voltages of V_{c1} and V_{c2} , which are used for controlling the flow rates of S_1 and S_2 , are of cosine profiles, and are independently varied over the range of 0–5 V with periods of 30 and 20 s, respectively. Analytical signals ($V_{d,Fe}$ and $V_{d,MB}$) are both acquired at a sampling frequency of 8.533 Hz. The least common multiple (60 s) of the V_{c1} and V_{c2} periods (30 and 20 s, respectively) is set as the length of the FFT window. Eight data in this window (*i.e.*, every sixty fourth 8 points) are used for FFT computation. FFT computation is carried out in real time (every 1.875 s) using a new set of 8 data by shifting the FFT window.

Results and Discussion

Selection of internal standard

The selection of the internal standard substance is of critical importance for applying the internal standard method. As for the spectrophotometric determination of Fe^{2+} in the present study, the internal standard substance must not interfere with the color reaction; both the analyte and the substance need to have no mutual spectral interference. The spectra of some dyes (Methylene Blue, Methyl Orange, Crystal Violet, Malachite Green, Erythrosine B, Acid Red S (Amaranth), Fast Green FCF, Indigo Carmine) were measured as candidates for the internal standard substance. Among them, Methylene Blue (MB) was found to be most suitable because both MB and the Fe^{2+} -*o*-Phen complex scarcely showed absorbance at each other's maximum absorption wavelength. It was confirmed that the color formation of the Fe^{2+} -*o*-Phen complex and its absorbance at 510 nm were not interfered with by $10 \mu\text{mol dm}^{-3}$ MB. MB was, therefore, selected as the internal standard substance in the present study. Some reports on the application of MB as an internal standard substance have been published. These include reversed HPLC,^{15,16} second-derivative spectrophotometry¹⁷ and micellar electrokinetic chromatography.^{18,19}

Flow signals

Figure 2(A) shows the typical flow signals of the internal standard-AMMFA. The samples (S_1 and S_2 in Fig. 1) are both aqueous solutions containing $50 \mu\text{mol dm}^{-3}$ Fe^{2+} and $10 \mu\text{mol dm}^{-3}$ MB. The amplitudes and periods of the control signals (V_{c1} and V_{c2}) are the same as those listed in Table 1. Thus, periodically varying flow rates of the samples result in changes of the Fe^{2+} and MB concentrations in the merged solution. The resulting analytical signals for Fe^{2+} ($V_{d,Fe}$) and MB ($V_{d,MB}$), therefore, show a periodic nature with a period of 60 s (the least common multiple of the periods of V_{c1} and V_{c2}), which is set as the length of the FFT window. There is a phase delay of the V_d signals behind the V_c signals. The reason for the delay is that V_c determines the composition of the solution at the confluence point, and there is a lag time between the upstream confluence and the downstream detection. The lag time estimated from the length of the arrow in Fig. 2A is 19.1 s.

Figure 2B shows the amplitudes obtained by FFT analysis for the data shown in Fig. 2A. Based on the relationships between the control periods (30 and 20 s for S_1 and S_2 , respectively) and the length of the FFT window (60 s), the amplitudes of the second and third-harmonic wave components correspond to the concentrations of the components in S_1 and S_2 , respectively. Hereinafter, A_{2Fe} and A_{2MB} denote the amplitudes of the second-harmonic wave components in V_d at 510 and 664 nm, corresponding to the concentrations of Fe^{2+} and MB in S_1 , respectively; similarly, A_{3Fe} and A_{3MB} mean the amplitudes of the third-harmonic wave components, corresponding to the concentrations of Fe^{2+} and MB in S_2 , respectively. The start of FFT computation needs to wait for 60 s until a complete data set for one FFT window is acquired. FFT is based on the assumption that the signals in the window continue infinitely as periodic signals. Therefore, if the duration time for each sample is less than 60 s, no reliable data can be obtained because of confliction with the FFT assumption. When n FFT data are needed for each amplitude, the duration time should be set at $(60 + 1.875n)$ s for each sample. The amplitudes obtained (mean \pm s.d., $n = 64$) are 0.2253 ± 0.0042 , 0.2804 ± 0.0061 , 0.1392 ± 0.0065 and 0.1687 ± 0.0084 V for A_{2Fe} , A_{2MB} , A_{3Fe} and A_{3MB} , respectively. FFT computation is carried out every 1.875 s using a new set of 8

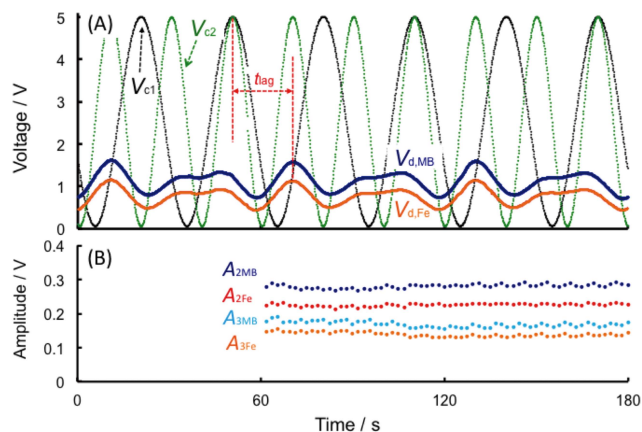


Fig. 2 (A) Typical flow signals of amplitude-modulated multiplexed flow analysis. (B) Amplitudes obtained by FFT analysis. Samples (S_1 and S_2 in Fig. 1) are both aqueous solution containing $50 \mu\text{mol dm}^{-3}$ Fe^{2+} and $10 \mu\text{mol dm}^{-3}$ MB. V_{c1} and V_{c2} , controller output voltages with the periods of 30 and 20 s, respectively; $V_{d,Fe}$, detector output voltage for Fe^{2+} ; $V_{d,MB}$, detector output voltage for MB. The window for FFT computation is 60 s. A_{2Fe} and A_{2MB} , amplitudes of second harmonic wave components of Fe^{2+} and MB, respectively. A_{3Fe} and A_{3MB} , amplitudes of third harmonic wave components of Fe^{2+} and MB, respectively. The arrow in Fig. 2A indicates the lag time between the control and detection, mainly corresponding to the physical transit of the fluid from the confluence point to the detector.

data, as described in the section concerning Software (also see Table 1). On the other hand, the 8 data for the FFT computation are picked up every 7.5 s ($= 64/8.533$ Hz). The phase of 8 data, therefore, come to be coincident every 4 ($= 7.5 \text{ s}/1.875$ s) FFT data. These facts are considered to be responsible for the very fine oscillations with a period of 7.5 s (4 FFT data) that can be seen in the amplitudes. The amplitudes of the second-harmonic wave components (A_{2Fe} , A_{2MB}) are higher than the corresponding ones of the third-harmonic wave components (A_{3Fe} , A_{3MB}) even though the same concentrations of Fe^{2+} and MB are, respectively, added in S_1 and S_2 . The reason for this difference is discussed in the next session. The ratios of A_{2Fe}/A_{2MB} and A_{3Fe}/A_{3MB} are 0.8034 ± 0.0060 and 0.8254 ± 0.0140 , respectively. The values of the relative standard deviation (RSD) for A_{2Fe}/A_{2MB} and A_{3Fe}/A_{3MB} are 0.74 and 1.70%, whereas those of A_{2Fe} and A_{3Fe} are 1.87 and 4.67%, respectively. These results suggest the advantage of the internal standard method over the absolute calibration method.

Analytical performance

Calibration curves were constructed from five standard solutions, each containing Fe^{2+} (0, 25, 50, 75 or $100 \mu\text{mol dm}^{-3}$) and MB (uniformly $10 \mu\text{mol dm}^{-3}$). The software parameters are the same as those listed in Table 1. Table 2A summarizes the analytical performances of the internal standard method and the absolute calibration method. It should be noted that the slopes of the absolute calibration curves are dependent on the control periods. That is, amplitude damping through dispersion in the conduit is more pronounced when the control period becomes shorter.² The linearity of the calibration curves obtained by the former method ($r^2 \geq 0.9997$) is better than those by the latter method ($r^2 \geq 0.9961$). The values of LOD (3.3σ) were greatly improved by introducing the internal standard method. The effect of the internal standard method is more pronounced for S_2 , the flow rate of which is modulated at a

Table 2 Calibration curves

Sample	Internal standard method				Absolute calibration method			
	Slope	Intercept	r^2	LOD/ $\mu\text{mol dm}^{-3}$	Slope	Intercept	r^2	LOD/ $\mu\text{mol dm}^{-3}$
(A) Without any deliberate fluctuation signal								
S ₁	0.0158	0.0208	0.9999	1.46	0.0044	0.0071	0.9999	2.18
S ₂	0.0164	0.0156	0.9997	3.05	0.0027	0.0062	0.9961	11.90
(B) With cosine waved fluctuation signal								
S ₁	0.0169	0.0556	0.9996	3.70	0.0032	0.0074	0.9989	6.27
S ₂	0.0174	0.0575	0.9994	4.55	0.0023	0.0002	0.9918	17.27
(C) With triangular waved fluctuation signal								
S ₁	0.0172	0.0156	0.9992	5.38	0.0030	0.0113	0.9930	15.98
S ₂	0.0175	0.0256	0.9992	5.40	0.0022	0.0093	0.9966	11.07

(A) Control signals contain no deliberate fluctuation signal. Flow rates of S₁ and S₂ are modulated at the periods of 30 and 20 s, respectively. (B) and (C) Control signals consist of original control signals and fluctuation signals. Original control signals are of cosine profiles (amplitude: 3–3.5 V) with the periods of 30 and 20 s for V_{c1} and V_{c2}, respectively. Fluctuation signals are of cosine profiles (amplitude: 0–1.5 V) with the periods of 60 and 120 s for V_{c1} and V_{c2}, respectively (B), or are of triangular profiles (amplitude: 0–1.5 V) with the periods of 120 and 90 s for V_{c1} and V_{c2}, respectively (C).

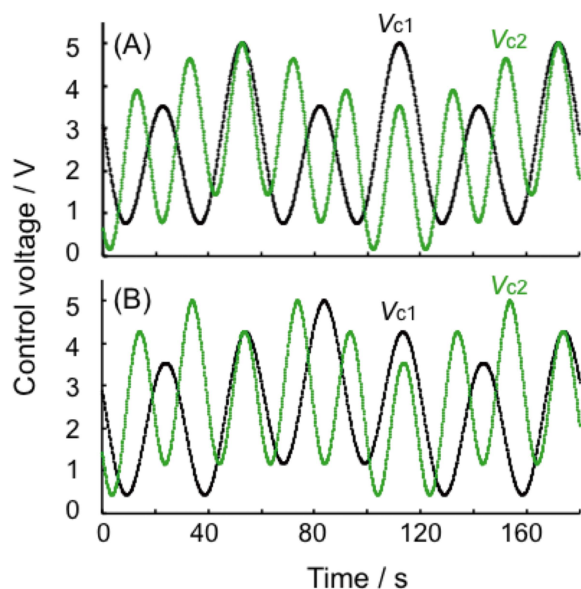


Fig. 3 Control signals for robustness test. (A) V_{c1}: Cosine wave (period, 30 s; amplitude, 0–3.5 V) + cosine wave (period, 60 s; amplitude, 0–1.5 V). V_{c2}: Cosine wave (period, 20 s; amplitude, 0–3.5 V) + cosine wave (period, 120 s; amplitude, 0–1.5 V). (B) V_{c1}: Cosine wave (period, 30 s; amplitude, 0–3.5 V) + triangular wave (period, 120 s; amplitude, 0–1.5 V). V_{c2}: Cosine wave (period, 20 s; amplitude, 0–3.5 V) + triangular wave (period, 90 s; amplitude, 0–1.5 V). The amplitude is expressed as peak to peak voltage.

higher frequency, and the resulting dispersion is more pronounced compared to S₁. That is, LOD is improved from 11.90 $\mu\text{mol dm}^{-3}$ (absolute calibration method) to 3.05 $\mu\text{mol dm}^{-3}$ (internal standard method).

Robustness of system

In order to verify the robustness of the internal standard-AMMFA against the undesired fluctuation in the flow rate, fluctuation signals were imposed on the original control signals, using a software supplied by the manufacturer of the signal generator.²⁰ Figure 3 shows typical examples of V_{c1} and V_{c2} that contain fluctuation signals of cosine profiles (A) or triangular wave profiles (B). Original control signals are of cosine profiles (amplitude, 0–3.5 V) with periods of 30 and 20 s for V_{c1} and

V_{c2}, respectively. Fluctuation signals are of cosine profiles (amplitude, 0–1.5 V) with periods of 60 and 120 s for V_{c1} and V_{c2} (A), or of triangular profiles (amplitude, 0–1.5 V) with periods of 120 and 90 s for V_{c1} and V_{c2}, respectively. The length of FFT window was held at 60 s, which corresponded to the least common multiple of the periods of the original control signals (30 and 20 s for V_{c1} and V_{c2}, respectively), although the fluctuation signals that have longer periods (*i.e.*, 90 and 120 s) than the length of the FFT window were contained in the control signals. Calibration curves were constructed using the five standard solutions mentioned above. Table 2 (B and C) summarizes the obtained calibration curves. Irrespective of the profiles of the fluctuation signals, the values of LOD were greatly improved by introducing the internal standard method. The linearity for the absolute calibration method is seemingly not much worse. It should be mentioned, however, the value of RSD for each plot could be greatly reduced by introducing the internal standard method. That is, the average values of RSD for the plots of the set (B) in Table 2 are 14.40, 16.73, 6.41 and 8.25%, and those for the plots of the set (C) are 15.78, 16.24, 5.20 and 7.28% for A_{2Fe}, A_{3Fe}, A_{2Fe}/A_{2MB} and A_{3Fe}/A_{3MB}, respectively (Supporting Information).

Stability of continuous operation

Figure 4 shows the validation results of the present system against the rather longer period of operation (1 h), where V_{d,Fe} and V_{d,MB} (A), A_{2Fe} and A_{3Fe} (B), and A_{2Fe}/A_{2MB} and A_{3Fe}/A_{3MB} (C) are plotted as a function of time. The concentrations of Fe²⁺ and MB were 50 and 10 $\mu\text{mol dm}^{-3}$, respectively, both for S₁ and S₂. As for the absolute calibration method (B), the values of A_{2Fe} and A_{3Fe} ($n = 1888$) were 0.233 ± 0.009 V (RSD = 4.18%) and 0.147 ± 0.009 V (RSD = 6.74%), respectively. The amplitudes sometimes fluctuated rather remarkably, probably due to accidental fluctuations of the flow rate. This fluctuation is greatly reduced by introducing the internal standard method (C). That is, the values of A_{2Fe}/A_{2MB} and A_{3Fe}/A_{3MB} were 0.799 ± 0.010 (RSD = 1.27%) and 0.824 ± 0.019 (RSD = 2.38%), respectively. These results indicate that the accidental fluctuation of the flow rate is greatly reduced by computing the ratio of the Fe²⁺ amplitude to the MB amplitude. The internal standard method can also reduce the effect of modulation period on the dispersion of the samples. The difference between the A_{2Fe}/A_{2MB} and A_{3Fe}/A_{3MB} values was *ca.* 3%. In contrast, A_{2Fe} (*i.e.*, 0.233 V) was 37% higher than A_{3Fe} (*i.e.*, 0.147 V).

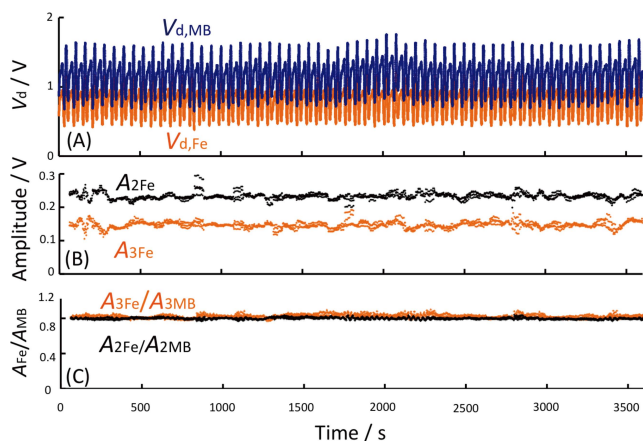


Fig. 4 Validation for the stability of the internal standard-amplitude modulated multiplexed flow analysis. (A) Detector output signals for Fe^{2+} and MB. (B) Amplitudes corresponding Fe^{2+} concentrations (absolute calibration method). (C) Ratios of the amplitude of A_{Fe} to that of A_{MB} (internal standard method). S_1 and S_2 are an aqueous solution of $50 \mu\text{mol dm}^{-3}$ Fe^{2+} spiked with $10 \mu\text{mol dm}^{-3}$ MB. The flow rates of S_1 and S_2 are modulated at the periods of 30 and 20 s, respectively. The window for FFT computation is 60 s. $V_{d,Fe}$ and $V_{d,MB}$ are the detector output voltages for Fe^{2+} and MB, respectively; A_{2Fe} and A_{3Fe} are the amplitudes of the second and third harmonic wave components in $V_{d,Fe}$, corresponding to the concentrations of Fe^{2+} in S_1 and S_2 , respectively; A_{2MB} and A_{3MB} are the amplitudes of the second and third harmonic wave components in $V_{d,MB}$, corresponding to the concentrations of MB in S_1 and S_2 , respectively.

Table 3 Application to real water samples ($n = 3$)

Sample	Recovery, %				
	Internal standard method		Absolute calibration method		
S_1	S_2	S_1	S_2	S_1	S_2
1	2	96.6 ± 1.1	96.2 ± 0.5	98.0 ± 4.7	99.1 ± 1.1
3	4	102.3 ± 0.8	98.9 ± 1.8	101.4 ± 4.1	101.3 ± 3.5
5	1	95.8 ± 1.2	97.1 ± 1.7	103.3 ± 7.2	106.7 ± 0.7
2	3	100.2 ± 0.1	105.3 ± 1.1	101.1 ± 2.8	102.8 ± 3.0
4	5	101.2 ± 0.4	96.1 ± 0.4	101.8 ± 0.4	101.4 ± 4.0

Sampled from 1, the Fukuroi Irrigation; 2, the Yoshino River; 3, the moat of Shozui Castle; 4, the Naka Pond; 5, hot spring in Tokushima Prefecture. Sampling date: April 15, 2016. Concentration of Fe^{2+} spiked is $50 \mu\text{mol dm}^{-3}$. Flow rates of S_1 and S_2 were modulated at the periods of 30 and 20 s, respectively.

Application to real samples

The proposed method was applied to analyses of real water samples. No Fe^{2+} was detected in the samples both by the proposed method and by the batch method,¹¹ meaning that the concentration of Fe^{2+} was too low to be detected. Recovery tests were therefore carried out by spiking a known concentration of Fe^{2+} ($50 \mu\text{mol dm}^{-3}$) to the sample solutions. The results are listed in Table 3. Measurements for each set were carried out three times, and the duration of each run was 180 s. Samples were replaced manually. Both the internal standard method and the absolute calibration method can give good recoveries around 100%. On the whole, the reproducibility of the former method ($n = 3$) is better than that of the latter.

Conclusions

An internal standard-amplitude modulated multiplexed flow analysis is proposed. The concept was evaluated by applying it to the determination of Fe^{2+} by 1,10-phenanthroline spectrophotometry. Methylene Blue was selected as an internal standard substance. The precision, linearity of the calibration curve, limit of detection, and the robustness against deliberate fluctuation of the proposed method were greatly improved by introducing the internal standard method. In addition, the effect of amplitude damping through dispersion is reduced, and thus the dependence of the slope of the calibration curve on the modulation period becomes less by using the internal standard method.

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Supporting Information

Calibration curves for Fe^{2+} obtained by AMMFA, the flow rates of which are deliberately fluctuated by sinusoidal (A, B) and triangular (C, D) signals. Calibration curves are constructed through the absolute calibration method (A, C) and the internal standard method (B, D). This material is available free of charge on the Web at <http://www.jscs.or.jp/analsci/>.

References

- H. Tanaka, T. Mima, M. Takeuchi, and H. Iida, *Talanta*, **2008**, *77*, 576.
- Y. Kurokawa, M. Takeuchi, and H. Tanaka, *Anal. Sci.*, **2010**, *26*, 791.
- K. Inui, T. Uemura, T. Ogusu, M. Takeuchi, and H. Tanaka, *Anal. Sci.*, **2011**, *27*, 305.
- T. Ogusu, K. Uchimoto, M. Takeuchi, and H. Tanaka, *Talanta*, **2014**, *118*, 123.
- K. Inui, H. Yoshida, M. Takeuchi, and H. Tanaka, *J. Flow Inject. Anal.*, **2015**, *32*, 5.
- H. Yoshida, K. Inui, M. Takeuchi, and H. Tanaka, *Anal. Sci.*, **2012**, *28*, 523.
- T. Uemura, T. Ogusu, M. Takeuchi, and H. Tanaka, *Anal. Sci.*, **2010**, *26*, 797.
- D. A. Skoog, F. J. Holler, and S. R. Crouch, "Principles of Instrumental Analysis", 7th ed., **2017**, Cengage Learning, Boston, 15.
- S. W. Gibb, R. F. C. Nanbhoura, and P. S. Liss, *Anal. Chim. Acta*, **1995**, *316*, 291.
- D. T. Gimenes, W. T. P. dos Santos, R. A. A. Munoz, and E. M. Richter, *Electrochem. Commun.*, **2010**, *12*, 216.
- JIS K 0102, "Testing Methods for Industrial Wastewaters", **2013**, Japanese Industrial Standard Comitee, Tokyo, Japan.
- K. Tsunoda, H. Itabashi, and H. Akaiwa, *Anal. Chim. Acta*, **1995**, *299*, 327.
- A. F. Oliveira, J. A. Nóbrega, and O. Fatibello-Filho, *Talanta*, **1999**, *4*, 505.
- S. Nakamura, "Beginner's Digital Fourier Transform", **1989**, Tokyo Denki University Press, 147 - 155.

15. A. El-Yazigi and A. Yusuf, *J. Pharmceut. Biomed.*, **1989**, 7, 877.
 16. K. T. Lin, G. E. Rivard, and J.-M. Leclerc, *J. Chromatogr. A*, **1989**, 465, 75.
 17. K. Wróbel, K. Wróbel, I. M. de la Garza Rodríguez, P. L. López-de-Alba, and L. López-Martínez, *J. Pharmceut. Biomed.*, **1999**, 20, 99.
 18. P. Jing, T. Kaneta, and T. Imasaka, *Electrophoresis*, **2002**, 23, 550.
 19. P. Jing, T. Kaneta, and T. Imasaka, *J. Chromatogr. A*, **2002**, 959, 281.
 20. NF Corp, www.nfcorp.co.jp/support/download/wf/1973_74_arb.html.
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