μSI: Optimization of Reagent Based Chloride Assay in Lab-on-Valve System

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Abstract

A spectrophotometric chloride assay has been investigated, employing an integrated lab-on-valve manifold in a micro fluidic sequential injection format (μSI). By optimizing the chemical and physical parameters a limit of detection of 45.02 ppb was obtained with r^2 = 0.9917 (0-1000 ppb). The assay for higher concentrations (0-100 ppm) was also achieved by microfluidic manipulation of the sample zone within the holding coil. Thus, this method is useful for chloride analysis over a 3-decade concentration range. The method described serves as a template for downscaling other colorimetric assays where addition of the analyte results in a change of absorbance but not the color.

Keywords μSI, lab-on-valve, chloride assay

1. Introduction

The chloride assay is one of the most important indexes of water quality, and many spectrophotometric techniques have been developed [1]. The Utsumi's method [2,3], using mercury(II) thiocyanate and iron(III) ion, is now widely applied to the chloride assay. This method is based on the following reactions.

$$Hg(SCN)_2 + 2Cl^- \rightarrow HgCl_2 + 2SCN^-$$
$$Fe^{3+} + SCN^- \rightarrow FeSCN^{2+}$$

This method was automated for clinical and industrial laboratories [4], and adapted to flow injection (FI) by utilizing the stream sample splitting technique [5,6]. This colorimetric method, however, has disadvantages in that a toxic reagent is needed and that the dark yellowish color of the reagent interferes with the detection of the red colored product.

Miniaturization of flow-based assays is at present an actively pursued topic in analytical research. Based on flow injection, sequential injection (SI) has been developed for reducing reagent and sample consumption [7,8]. The SI system consists of the central processing unit (lab-on-valve manifold), the syringe pump, holding and mixing coil, and the detector. In a conventional FI system, samples are injected into a continuous flow that is always generating waste. The sample and reagent solutions in a SI system, are sequentially aspirated only as needed, into the lab-on-valve manifold, and then are dispensed to the detector using a minimal of carrier solution. Thus, the SI method can substantially reduce waste production and reagent consumption. Another advantage to the SI system is that different reagent based assays can be performed using the same hardware structure, since variations in the experimental protocols (sample size, flow rates, mixing periods) are programmable. The greatest advantage of the SI method is its ability to handle wider dynamic concentration ranges through programmable dispersion. There are several reports [8,9] and reviews [10,11] for analysis using SI system. The extension of the SI system to the chloride assay with the Utsumi's method may be expected to reduce the amount of the toxic reagent, achieve lower detection levels and handle a wider concentration range. The analytical protocol described in this work will also be applicable to other SI analyses where the reagent has high background color.

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2. Experimental

2.1 Instrument

The sequential injection system (FIAlab-3000, FIAlab Instruments, http://www.flowinjection.com) consists of a stepper motor driven syringe pump (1000 μL), a six-port selector valve, and a single speed unidirectional peristaltic minipump, as shown in Figure 1. A tungsten-halogen lamp (LS-1, Ocean Optics Inc.,

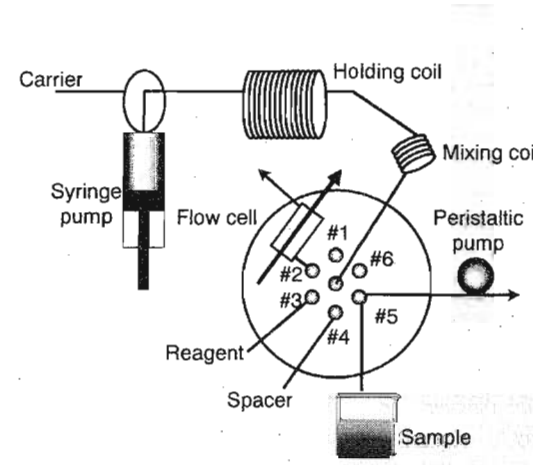

Figure 1. Schematic layout of the μSI instrument with a precision syringe pump (1000 μL) connected to the holding coil and the mixing coil. These coils are where the stacked zone of reagent/sample is made. The lab-on-valve device was mounted atop a six-way, multi-port valve, which allows for the fluidic operations of sample (port #5) and reagent (port #3) selections as well as the selection of the flow cell (port #2). An auxiliary peristaltic pump serves as the flow-through port (port #5) for rapid sample exchange. The flow cell is shown in absorbance configuration using two optical fibers facing each other. The internal diameter (I.D.) of all the tubing was 0.5mm (O.D.: 1.6mm).

http://www.oceanoptics.com) was used as the VIS light source for the UV-VIS spectrophotometer (USB2000, Ocean Optics Inc., http://www.oceanoptics.com). Fiber optic cables, furnished with a stainless steel clad tip (400 micron, tip 0.0625'' OD) were used to connect the flow cell to the light source and the spectrometer. Data was collected by FIAlab for Windows 98 (version 5.9.30) installed on a PC (Pentium II 300 MHz, 64 MB RAM) with Windows 98 (Microsoft Inc., http://www.microsoft.com) as the operating system.

The lab-on-valve manifold was fabricated in-house and interfaced to match a six-port selector valve as described earlier [8]. The central inlet was connected to a holding coil. The flow cell was designated to connect port #2 of the six-port valve to the integrated flow through detector. The sampling port was set to port #5 while the waste was designated to #1, the reagent was designed to #3, and spacer was designated to #4. The flow cell was configured for absorbance measurements at 460 nm, as shown in Figure 1, and the pass length was set with 10 mm for low concentration (0-1000 ppb) assay or 5 mm for high concentration (0-100 ppm) assay.

2.2 Reagents and standards

A 100 ppm chloride stock solution was made by dissolving sodium chloride (J.T. Baker Inc., http://www.jtbaker.com) in DI water, and then the standard solutions were made by dilution from this stock solution. The reagent solution was made by dissolving appropriate amount of iron(III) nitrate nonahydrate (Fisher Scientific USA, http://www.fishersci.com), mercury(II) thiocyanate (Sigma USA, http://www.sigma-aldrich.com), nitric acid (Fisher Scientific) and methanol (Fisher Scientific) into the DI water to a final volume of 100 mL. DI water was used for both carrier and spacer. All assays were carried out triplicate and the average value was used for the calibration.

3. Results and Discussion

3.1 μSI protocol

The μSI technique allows sample and reagent solutions to be selected, mixed, and diluted automatically. This is achieved by sequentially aspirating, from the multi-port valve, sample and reagent into stacked zones into a holding coil (Figure 1). It has been shown that it is preferable to inject the sample zone first, followed by the reagent zone in the sequence required by the chemistry [7]. To promote good sample/reagent zone overlap and mixing, an additional spacer zone of carrier solution is aspirated into the holding coil. In this study, the μSI method starts by stacking the zones into the holding coil in the following order: carrier, reagent, sample, and spacer. Following the delay time of 10 sec for the reaction, the composite zone was pushed into the detector cell. These series of executions are controlled simply by the software, as shown in Table 1. Figure 2(a) shows the typical peak shapes obtained with the protocol shown in Table 1(a). There is another possible protocol which uses the reagent as the spacer instead of DI water. Figure 2(b) shows the typical peak shapes obtained when the reagent was used as the spacer. In this case, the product peak is hidden due to the large absorbance of the reagent, and the concentration cannot be calibrated. As shown, the background of the reagent during the protocol should always be considered carefully, when the reagent has similar color to the product.

3.2 Optimization of the reagent and the protocol

The reagent concentration was changed and its effect on the slope of the calibration curve in low sample concentration region (0-1000 ppb) was investigated in order to optimize the assay for the highest sensitivity. In this case, the light pass was set as 10 mm, the aspiration rate for reagent, sample, and spacer was set as 50 μL/sec, and the integration time of the spectrophotometer was set as 500 msec. Figure 3 shows the effect of the concentration of iron(III) nitrate and mercury(II) thiocyanate on the slope of the calibration curve. For both iron(III) nitrate and mercury(II) thiocyanate, the sensitivity is greater with increased reagent concentration. The improvement of the sensitivity is no longer obtained around 1.2 M of iron(III)

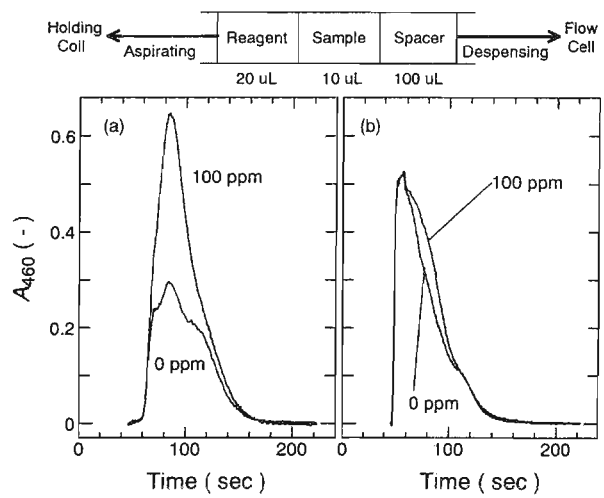

Figure 2. The peak shape obtained for the blank control and the 100 ppm sample. The stacked zones of sample, reagent, and spacer are depicted for the chloride assay. Spacer is (a) water or (b) reagent. Light pass and integration time are (a) 5 mm and 500 msec, and (b) 1 mm and 200 msec.

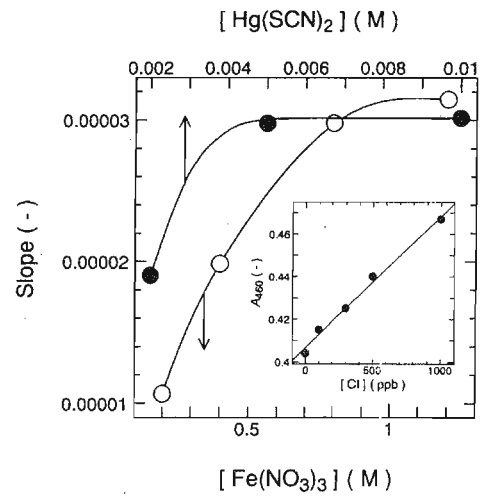

Figure 3. The effect of the concentration of iron(III) nitrate and mercury(II) thiocyanate on the slope of the calibration curve. The volumes of the injected zones are 50 μL (sample), 10 μL (reagent), and 50 μL (spacer). Inset: Typical calibration curve of chloride in low concentration region. The volumes of each injected zone are 100 μL (sample), 20 μL (reagent), and 50 μL (spacer). Light pass is 10 mm.

Table 1. μSI protocol[a] in FIALab™.

Protocol	Note	Protocol	Note
Syringe Pump Command (?) K0R	Turn off backlash compensation of syringe pump	(b) Delay (sec) 1	Delay 1 sec for the reaction
Peristaltic Pump On Delay (sec) 10 Peristaltic Pump Off	Cleaning and reagent preparation	Multiposition Valve Flow Cell Syringe Pump Flowrate (μL/sec) 10 Syringe Pump Dispense (μL) 70 Syringe Pump Delay Until Done	Dispense the solutions to flowcell
Loop Start (#) 3	Repeat the same assay three times	Spectrometer Reference Scan Spectrometer Absorbance Scanning	Start the ref. and abs. scan
Syringe Pump Valve In Syringe Pump Flowrate (μL/sec) 100 Syringe Pump Aspirate (μL) 400 Syringe Pump Delay Until Done Syringe Pump Valve Out	Aspiration of carrier solution	Syringe Pump Flowrate (μL/sec) 3 Syringe Pump Dispense (μL) 80 Syringe Pump Delay Until Done Syringe Pump Flowrate (μL/sec) 100 Syringe Pump Empty Syringe Pump Delay Until Done Spectrometer Stop Scanning	
Multiposition Valve Reagent Syringe Pump Flowrate (μL/sec) 10 Syringe Pump Aspirate (μL) 20 Syringe Pump Delay Until Done	Aspiration of reagent solution	Continued to (c)	
Multiposition Valve Sample Syringe Pump Flowrate (μL/sec) 10 Syringe Pump Aspirate (μL) 10 Syringe Pump Delay Until Done	Aspiration of sample	(c) Syringe Pump Valve In Syringe Pump Flowrate (μL/sec) 200 Syringe Pump Aspirate (μL) 500 Syringe Pump Delay Until Done Syringe Pump Valve Out Multiposition Valve Flow Cell Syringe Pump Empty Syringe Pump Delay Until Done	Restoring the initial condition of the flowcell using high flow rate and flushing with additional carrier solution
Multiposition Valve spacer Syringe Pump Flowrate (μL/sec) 10 Syringe Pump Aspirate (μL) 100 Syringe Pump Delay Until Done	Aspiration of spacer		
Continued to (a) or (b)		Loop End	
(a) Delay (sec) 10	Delay 10 sec for the reaction		
Spectrometer Reference Scan Spectrometer Absorbance Scanning	Start the ref. and abs. scan		
Multiposition Valve Flow Cell Syringe Pump Flowrate (μL/sec) 3 Syringe Pump Empty Syringe Pump Delay Until Done Spectrometer Stop Scanning	Dispense the solutions to flowcell		
Continued to (c)			

[a] Actual experimental protocol

nitrate and 0.01 M of mercury(II) thiocyanate. In the case of iron(III) nitrate, concentrations above 0.8 M can cause air bubbles to form during the aspiration of the reagent solution, since the reagent solution becomes viscous. The effect of methanol and nitric acid in the reagent solution was also investigated. In these cases, the amount of methanol was changed from 40 to 70 mL, and that of the nitric acid was changed from 2 to 7 mL in 100 mL of the reagent solution. For both cases, the smaller amount gave good sensitivity. However, mercury(II) thiocyanate is difficult to dissolve in methanol volumes less than 40 mL. Based on these results, the reagent solution containing 0.8 M of iron(III) nitrate, 0.01 M of mercury(II) thiocyanate, 2 mL of nitric acid (in 100 mL of the reagent), and 50 mL of methanol (in 100 mL of the reagent) was used hereafter.

The effect of the volume of each injected reagent zone, sample and spacer, was also investigated. In the case of both reagent and sample, the slope of the calibration curve increased with increase in each injected volume, where 20 μL of reagent and 100 μL of sample gave good sensitivity. In the case of the spacer, the sensitivity increases with injected volume to ~50 μL, where it then decreases for volumes greater than 50 μL. This is due to excess sample and reagent dispersion since the spacer creates a longer path-length to the detector. Therefore, the

protocol with 20 μL of the reagent, 100 μL of sample, and 50 μL of spacer was used hereafter. The effect of physical parameters was finally investigated, for improvement the limit of the detection (LOD) value. The LOD value was defined as $3\sigma_b/m$, where σ_b is the standard deviation of the background signal and m is the slope of the calibration curve. The LOD value with above condition is 371.2 ppb. The LOD value improves to 104.0 ppb by decreasing the aspiration speed of each fragment from 50 μL/sec to 10 μL/sec, due to the greater aspiration precision possible. The LOD value also improves to 45.03 ppb with increasing integration time of the spectrometer to 2000 msec, although the data collection speed was compromised. The calibration curve obtained here is shown in the inset of Figure 3. A good linear relationship of $Y = 6.150\times10^{-5}X + 4.068\times10^{-1}$ where $r^2 = 0.9917$ was obtained.

3.3 Calibration of chloride

Based on the above studies, the chloride assay was carried out with the detection zone setup in the *"high concentration"* mode. In this case, the light path was set to 5 mm, and the integration time of the spectrometer was set to 500 msec. The aspiration rate of each zone was set as 10 μL/sec. Figure 4 shows the calibration curves obtained by changing the volumes of sample and spacer, while the injected reagent volume remained fixed at 20 μL. In the case of (a) 100 μL of sample and 50 μL of spacer, the calibration curve deviates from the linear relationship at around 25 ppm chloride. This deviation appears to be due to the limitation of Beer's law. The range where the linear relationship can be obtained thus increases to 50 ppm in the case of (b) 20 μL of sample and 100 μL of spacer, and to 100 ppm in the case of (c) 10 μL of sample and 100 μL of spacer, by the improved dispersion of the sample with the spacer. This is one of the advantages to the SI system, sample dilution can be achieved easily by changing the ratio of sample to spacer.

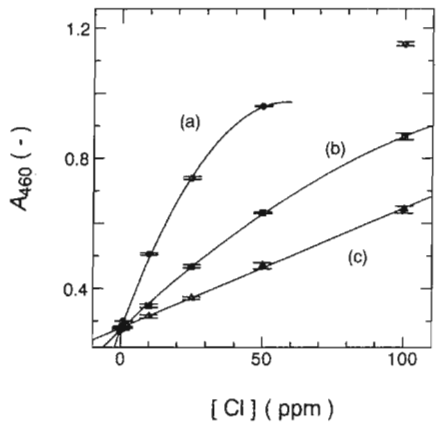

Figure 4. The calibration curve of chloride in the high concentration region. The stacked zones used were (a) 100 μL (sample), (b) 20 μL (sample), and (c) 10 μL (sample), 20 μL (reagent), and (a) 50 μL (spacer) and (b) & (c) 100 μL (spacer). Calibration curves obtained were:

(a) $Y = -2.050\times10^{-4}X^2 + 2.392\times10^{-2}X + 2.871\times10^{-1}$
$r^2 = 0.9995$ (0-50 ppm).

(b) $Y = -2.351\times10^{-5}X^2 + 8.286\times10^{-3}X + 2.741\times10^{-1}$
$r^2 = 0.9997$ (0-100 ppm).

(c) $Y = 3.682\times10^{-3}X + 2.793\times10^{-1}$
$r^2 = 0.9992$ (0-100 ppm).

One of the difficulties of this system is that the absorbance of the background is high, since the color of the reagent itself is similar to the product. For decreasing background absorbance, it is effective to set the reference scan (100% Transmittance) after 70 μL of the mixed solution is dispensed. The calibration curve obtained with this protocol is shown in Figure 5(a). The linear relationship between the concentration and absorbance is also obtained, at a cost to reproducibility. The sampling rate for each assay is also one of the important parameters. There are three possible ways to improve the sampling rate; (1) increase the flow rate immediately after peak identification, (2) decrease the delay time for the reaction, and (3) increase flow rate prior to reference scan. The protocol used is shown in Table 1(b). Figure 5(b) shows the calibration curve obtained with this protocol. A linear relationship is also obtained. Compared to Figure 5(a), higher background absorbance is

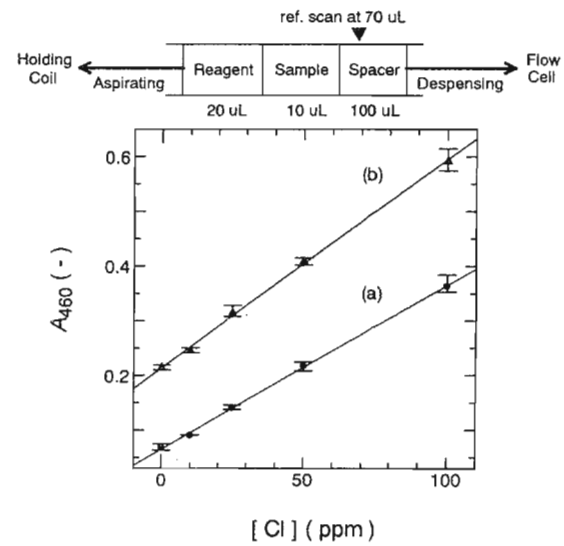

Figure 5. The calibration curves based on the reference scan with the spacer. The stacked zones of 10 μL of sample, 20 μL of reagent, and 100 μL of spacer (DI water) are depicted. The dispense rate until reference scan is (a) 3 μL/sec and (b) 10 μL, and the delay time for the reaction is (a) 10 sec and (b) 1 sec.

(a) $Y = 2.990\times10^{-3}X + 6.562\times10^{-2}$, $r^2 = 0.9995$.

(b) $Y = 3.803\times10^{-3}X + 2.145\times10^{-1}$, $r^2 = 0.9988$.

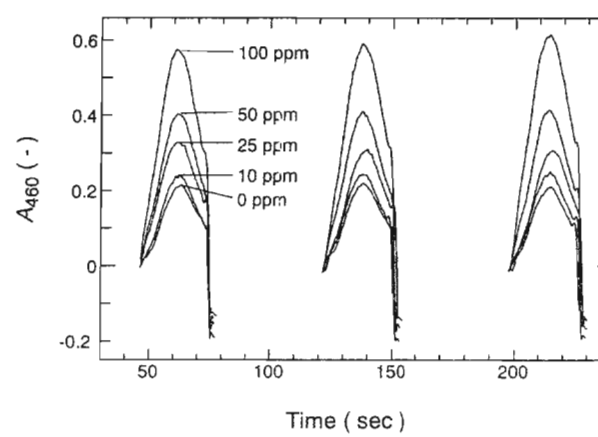

Figure 6. The actual peak shape for the Figure 5(b).

observed because of limited reagent dispersion due to the short delay time. In this case, the reproducibilities are also decreased, and the relative standard deviations increase about 1.5~3 times compared to those of Figure 4(c). Figure 6 shows the actual peaks obtained by the same protocol for Figure 5(b). The sampling rate is improved and a throughput frequency of 57 samples/h can be obtained, while it was 17 samples/h with the protocol shown in Table 1(a).

4. Conclusion

The chloride assay with the sequential injection system has been investigated, with the following results. The determination of chloride both in the low and high concentration region can be carried out. Indeed, this method is useful for chloride analysis over a 3-decade concentration range, while most colorimetric assays can only be performed over 1-2 decades. In the case of a low concentration region, a limit of detection value of 45 ppb was obtained with the appropriate run conditions. In the case of a high concentration region, chloride can be determined by increasing sample dispersion, which is easily achieved by changing the volumetric ratio of sample to spacer. This μSI approach reduces the use of the toxic reagent to just 20 μL/sample, which is more than a 200-fold reduction to conventional FIA techniques [5] and more than a 50-fold reduction to the original Utsumi's method [2].

Acknowledgement

S.N. is grateful to the Research Fellowships of the Japan Society for Promotion of Science for Young Scientists and to the Ogasawara Foundation for the Promotion of Science and Engineering.

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(Received January 9, 2002)
(Accepted February 28, 2002)