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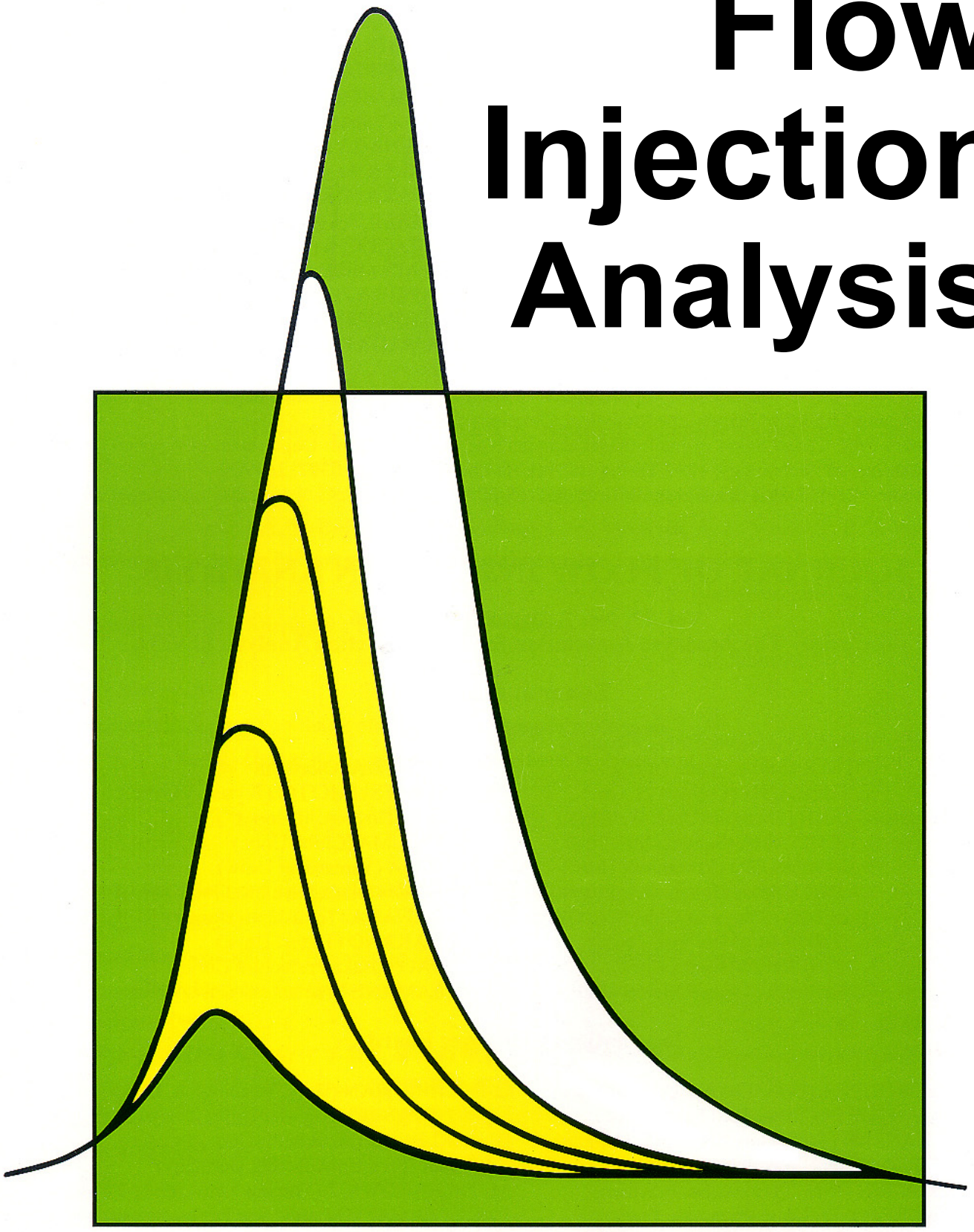

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Principles of micro Sequential Injection Analysis in the Lab-on-Valve format and its Introduction into a Teaching Laboratory

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Abstract

Advantages of programmable flow for optimization and automation of assay protocols in the lab-on-valve format are summarized and demonstrated in two laboratory experiments designed for a teaching or research laboratory as a tool for dissemination of this powerful analytical technique.

Keywords

Sequential Injection**,** programmable flow, spectrophotometry, phosphate, glucose, enzymatic assays, lab-on-valve

1. Introduction

The purpose of this paper is to summarize the principles and highlight the applications of the micro Sequential Injection (μSI) technique, and to facilitate its introduction into teaching and research laboratories. This will allow present users of Flow Injection Analysis (FIA) to proceed to a higher technology level since μSI offers improved versatility, time and reagent savings, and significant reduction of the volume of laboratory waste. The experiments included in this tutorial have been designed to facilitate understanding of the basics of flow programming, a technique that represents the principal technological advance from classical FIA. Indeed, by operating on programmable rather than continuous flow, the assay protocols can be optimized by selecting such flow rates and microfluidic manipulations that are suited to the needs of individual steps of assay protocols.

Initially designed as a tool for process control monitoring [1], Sequential Injection (SI) has found application in many fields for a wide variety of assays, including food and beverage analysis, bioprocess monitoring, pharmaceutical analysis and immunoassays [2]. However, it surpassed the established FIA methodologies (that now comprise over 16,000 papers [3]), only when it became miniaturized in the "lab-on-valve format. By integrating manifold components (flow cell, sampling port, separation columns, enzyme reactors, etc.) into a single, monolithic structure, mounted atop a conventional multiposition valve, the internal volume of the analytical flow path was downscaled to microliter volumes [4]. The resulting technique, microSI in Lab-on-valve format (μSI-LOV), is rapidly being accepted worldwide [5], because it 1) allows downscaling of current FIA and SI techniques,2) accommodates new analytical techniques such as Bead Injection, microAffinity Chromatography and Sequential Injection Chromatography, and 3) facilitates automation of spectrophotometry, atomic spectroscopies, chemiluminiscence, flourescenece and electrochemical techniques [5,6].

Micro Sequential Injection uses a high-precision syringe pump and a multiposition valve (Fig. 1) and *programmable flow* to mix and transport zones of reagent and sample in to a detection cell for analysis. Sample solution (Fig. 1, top (A)) is aspirated by the syringe pump, via the multiposition valve, into a holding coil. Next (B), the multiposition valve is switched to a reagent position and the first reagent is also aspirated into the holding coil. In the third step (C), the multiposition valve is switched to a second reagent or carrier reservoir and a volume is aspirated into the holding coil behind the first reagent zone, forming three stacked, partially intermixed zones. Next (D), the multiposition valve is switched to the detector port (#2) and the forward flow is accelerated, mixing and transporting the reaction mixture into the flow cell. The progress of sample/reagent reaction is monitored as reaction rate while the forward flow is stopped (E) and the stacked zones are arrested in the flow cell. Finally the flow cell is flushed by restarting the forward flow. Typical sample and reagent volumes are 10 μL and 40 μL, respectively, and the flow rates are selected to suit the analytical protocol (Tables 1 and 2). The syringe is filled at high flow rate, sample and reagents are carefully metered at low flow rates, reaction rate is monitored at stopped flow, and the system is flushed at a very high flow rate. More advanced methodologies, such as Bead Injection or microSI Chromatography, use appropriate flow rates for column packing (20 μL/sec), for column perfusion (1 μL/sec), and for discarding of stationary phase (200 μ.L/sec). Yet another advantage of programming the flow is improved reagent economy and reduction of the volume of chemical waste, compared with techniques based on continuous reagent pumping. The μSI-LOV apparatus (Fig.1. bottom) is compact, has only two moving parts, and all of the system functions, including spectrophotometry and data evaluation are software controlled. In teaching laboratories, the robustness of the system and low reagent consumption have obvious practical advantages. However, the main purpose of focusing on μSI-LOV in this paper is to facilitate the introduction of this versatile tool to research and teaching laboratories that currently use traditional Flow Injection techniques.

2. Experimental.

2.1. Apparatus

The micro Sequential Injection system was comprised of a FIAlab 3000 instrument furnished with a 500 μL syringe, a 900 μL holding coil (made of 0.8 mm I.D. Teflon tubing), and the LOV sample processing unit, mounted atop the six-port multiposition valve (FIAlab Instruments, Inc, www.flowinjection.com). A UV-Vis spectrophotometer (PC2000, Ocean Optics, Inc. http://www.oceanoptics.com) was used in all experiments. The light source used in this work was a Tungsten lamp, although a white light emitting diode, would serve equally well. The spectrophotometer and light source were connected to the flow cell, integrated within the LOV module,

by two fused silica optical fibers, the tips of the fiber optic cables being positioned 11 mm apart, creating the 22 µL flow cell. The system was controlled by a personal computer running FIAlab software version. 5.9.177.

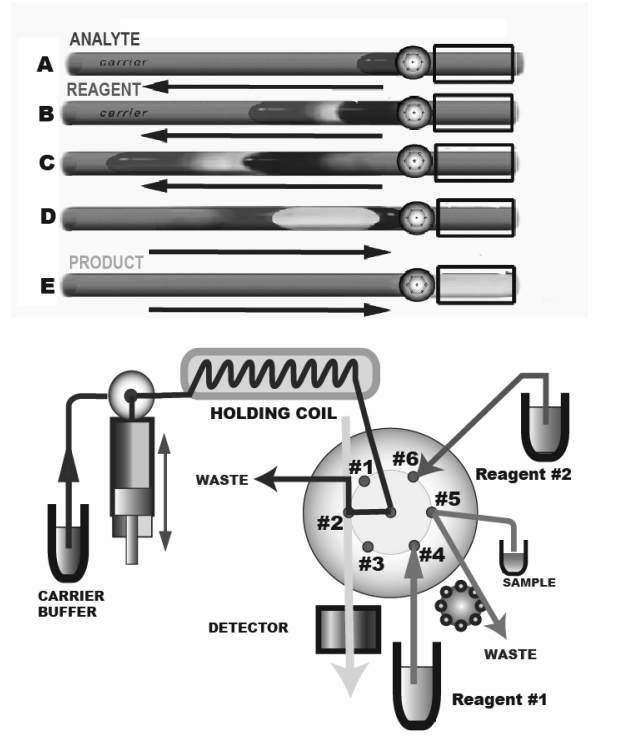

Fig. 1 (top): Principle of Sequential Injection**. Fig.1. (bottom):** Configuration of µSI-LOV system. The apparatus comprises a 500 µL high-precision, bi-directional syringe pump leading to a 900 µL holding coil and the Lab-on-Valve unit mounted atop a 6-port selector valve. The flow cell is contained within the LOV unit with the ends of the fiber optic cables spaced 11mm apart, creating a 22 µL flow cell. (Reproduced with permission from ref. 6).

2.2. Experiments

2.2.1. Determination of Phosphate – a two-reagent, stopped-flow assay

As one of the main nutrients required for plant survival, phosphates are important components in soil and fertilizers. Phosphates can also be found in bodies of water, such as lakes and ponds and streams. Household phosphates are found in a variety of products including carbonated drinks and laundry and dishwashing detergents. The prevalence of phosphates and the need to monitor them to ensure safe levels are the reasons why assays of phosphate are routinely performed, often using Flow Injection Analysis [3,7]. For a teaching laboratory, suitable samples for analysis could be diluted waste water from the washing machine or dishwasher or sampling from a local stream. For soil analysis this technique is applicable to soil extracts prepared in a routine manner.

The well known assay is derived from a manual procedure that relies on the formation of phosphomolybdenum blue, a colloidal solution that can be detected by monitoring absorbance at 620nm [8]. The reaction occurs according to the following reactions:

$$H_3PO_4 + 12H_2MoO_4 \rightarrow H_3P(Mo_{12}O_{40}) + 12H_2O$$

$$Mo(IV) \xrightarrow{\text{Ascorbic Acid}} Mo(V)$$

2.2.2. Reagents and Standards

A 6.9 g/L solution of ammonium molybdate (277908, Aldrich, www.sigmaaldrich.com) was prepared in 250 mL of DI water. An 80 g/L solution of ascorbic acid (A92902, Aldrich) was prepared in 250 mL of DI water and combined with an equal volume of a 0.05 g/L solution of potassium antimony (III) tartrate ($KSbOC_4H_4O_6.1/2\ H_2O$) (P6949, Sigma, www.sigmaaldrich.com). The ascorbic acid solution should be prepared daily. The carrier solution was citrate buffer (pH 3, 0.04 g trisodium citrate·$2H_2O$ and 0.18 g citric acid·$1H_2O$ in 100 mL H_2O (Sigma) containing 0.01% Brij (430AG-6, Sigma) as a surfactant. Orthophosphate standards were prepared by dissolving 0.220 g of anhydrous KH_2PO_4 in 50 mL of DI water and then performing serial dilutions of this stock. Standards ranged from 25 ppm to 200 ppm.

There are two important points that need to be made. First, when premixed, the reagents are incompatible, slowly forming a molybdenum blue color in the absence of phosphate. Therefore, reagents must be injected separately, in a programmed sequence that ensures a low blank value. The two-reagent, stopped-flow program fulfills this requirement (Table 1). Second, since the reduction of molybdate is slow, antimony tartrate is used to catalyze the reaction. The reaction rate is also pH dependent, so the reaction is performed under acidic conditions (pH 3). The example program is designed to aspirate 10µL of sample (port 5), 40 µL of molybdate reagent (port 6) and 60 µL of the ascorbic acid reducing solution (port 4) into the holding coil. The resulting stacked zones of analyte and reagents are then pushed into the flow cell and the flow is stopped for 13 seconds, while absorbance readings are taken by the spectrophotometer. As the zones are transported into the holding coil and then, using reversed flow, mixed and sent into the detector, phosphomolybdate is formed before it reacts with the ascorbic acid/tartrate solution. The production of colloidal phosphomolybdate blue over time allows for a plot of absorbance versus time to be generated (Figure 2). Calibration data collected from triplicate runs of four different concentrations of phosphate and a blank are shown in Figure 3. A plot of the initial reaction rates versus the phosphate concentration yields a calibration curve that is linear to 200 ppm (rate = 2.99×10^{-4} * [phosphate] + 9.66×10^{-3} with $R^2 = 0.941$). The limit of detection based on 3 standard deviations of the blank is 3.01 ppm phosphate.

Table 1: Phosphate µSI-LOV protocol

Hardware Settings Wavelength 1 (nm) 620

Spectrometer Reference Scan
Spectrometer Absorbance Scanning

Loop Start (#) 3

'Start-up
SyringePump Flowrate (microliter/sec) 100
SyringePump Valve In
SyringePump Fill
SyringePump Delay Until Done

'System wash
Valve port 2
SyringePump Valve Out
SyringePump Dispense (microliter) 150
SyringePump Delay Until Done

'Inject sample
Valve port 5
SyringePump Flowrate (microliter/sec) 10
SyringePump Aspirate (microliter) 10
SyringePump Delay Until Done

'Inject reagent 1
Valve port 4
SyringePump Flowrate (microliter/sec) 50
SyringePump Aspirate (microliter) 40
SyringePump Delay Until Done

'Inject reagent 2
Valve port 3
SyringePump Aspirate (microliter) 60
SyringePump Delay Until Done

'Send reactants into the flow cell
Spectrometer Reference Scan
Valve port 2
SyringePump Flowrate (microliter/sec) 75
SyringePump Dispense (microliter) 90
SyringePump Delay Until Done

'Stop flow and monitor
Delay (sec) 13

'Wash out the flow cell
Valve port 2
SyringePump Empty
SyringePump Delay Until Done

'End loop
Loop End

'Shut off spectrometer after last loop
Spectrometer Stop Scanning

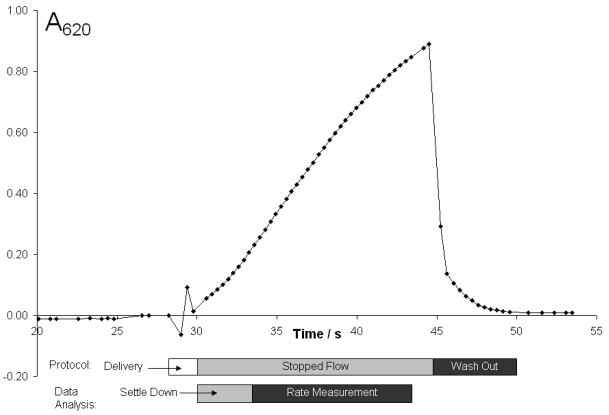

Figure 2: Absorbance monitoring, as recorded during the arrival of the reaction zone into the flow cell, the subsequent stopped-flow period, and the final washout. The linear rise of absorbance during stopped flow is used for system calibration.

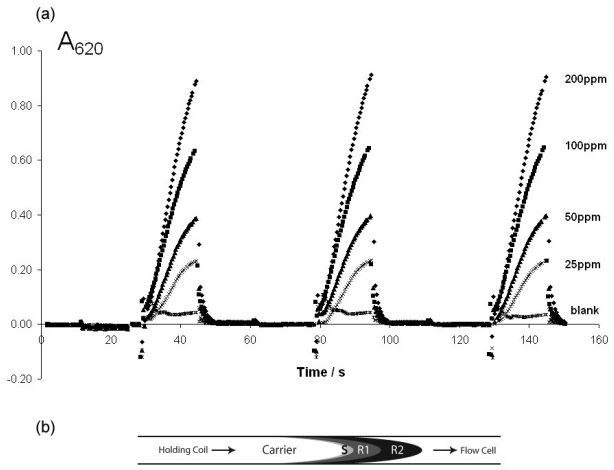

Figure 3: Stopped-flow assay of phosphate. (a) The assay of phosphate was performed in the range of 25-200 ppm with 3 replicate runs for each concentration shown superimposed. Data collection was performed continuously, with a reference scan just prior to the delivery of 90 µL dispensed volume. (b) The stacked zones consisted of 10 µL sample (S), 40 µL molybdate solution (R1) and 60 µL ascorbic acid solution (R2).

2.3.1. Determination of Glucose – a one-reagent, stopped-flow assay

Glucose, a simple monosaccharide sugar, is one of the most important carbohydrates and is used as an energy source by both plants and animals. The natural form of glucose (D-glucose) is produced by plants in a process called photosynthesis and is sometimes referred to as dextrose. In the human body, glucose is used in a process called glycolysis to produce ATP, the energy carrier of cells. Glucose, like phosphate, is vital to life. There are many uses for glucose assays [8], such as measuring blood sugar to detect diabetes or to perform quality control analyses on beverages.

The assay of glucose used in this work is based on the following enzymatic reactions:

$$\text{D-glucose} + H_2O \xrightarrow{\text{Glucose Oxidase}} H_2O_2 + \text{D-gluconate}$$

$$H_2O_2 + \text{4-aminoantipyrine} + \text{hydroxybenzoate} \xrightarrow{\text{Peroxidase}} \text{quinoneimine dye} + H_2O$$

The first step of the reaction involves the oxidation of glucose, a reaction that is catalyzed by glucose oxidase and produces hydrogen peroxide. The second step of the reaction, catalyzed by horseradish peroxidase, produces quinoneimine dye, which is pink in color and can be detected by monitoring the absorbance at 500 nm.

2.3.1. Reagents and Standards

One vial of dry glucose oxidase reagent (G7519-5L, Pointe Scientific, Inc. http://pointescientific.com/) was dissolved in 250 mL DI water prior to use. The reconstituted reagent contained >60 U/mL glucose oxidase, 4.8 U/mL peroxidase (Horseradish), 1.52 mM 4-aminoantipyrine, 40 mM p-hydroxybenzoate, pH 7.5 +/- 0.1 phosphate buffer and 0.4% sodium azide as a preservative. The carrier solution was DI water.

Glucose standards ranging from 125 ppm to 1000 ppm were made by performing serial dilutions from a 2000 ppm stock solution of D-glucose (G-8270, Sigma, www.sigmaaldrich.com). A possible sample for analysis could be fruit juice, which may require dilution in order to be within the calibration range.

The assay was performed at room temperature, which was sufficiently stable at 23 °C that thermostating of the apparatus was not necessary. For this assay, 50 µL of the glucose sample solution and 80 µL of the glucose oxidase reagent were stacked into the holding coil, followed by a spacer composed of 100 µL of carrier solution (Software protocol,Table 2).

Note that the inclusion of a spacer and the injection of reagent and spacer zones at high flow rates promote mixing in the axial direction (the spacer displaces the stacked glucose/reagent zones further upstream). The mixed zones were then sent to the flow cell by flow reversal and the flow was stopped when the zones were in a position where the largestresponse had been observed [6,10] (delivering a volume of 210 µL). The position of the reacting zone within the detector is defined by the delivered volume of flow reversal and must be experimentally determined, as discussed briefly in the next section. The calibration curve for the glucose assay was obtained from the initial slope of the reaction rate curve (Figure 4).

Table 2: Glucose µSI-LOV protocol

Hardware Settings Wavelength 1 (nm) 500

Loop Start (#) 3

'Aspirate carrier
SyringePump Valve In
SyringePump Flowrate (microliter/sec) 100
SyringePump Aspirate (microliter) 250
SyringePump Delay Until Done
SyringePump Valve Out

'Inject sample
Valve port 5
SyringePump Flowrate (microliter/sec) 30
SyringePump Aspirate (microliter) 30
SyringePump Delay Until Done

'Inject reagent
Valve port 4
SyringePump Flowrate (microliter/sec) 80
SyringePump Aspirate (microliter) 80
SyringePump Delay Until Done

'Inject spacer
Valve port 6
SyringePump Aspirate (microliter) 100
SyringePump Delay Until Done

'Send reactants into the flow cell
Valve port 2
SyringePump Dispense (microliter) 210
SyringePump Delay Until Done

'Settle down period
Delay (sec) 15

'Data collection
Spectrometer Reference Scan
Spectrometer Absorbance Scanning
Delay (sec) 20
Spectrometer Stop Scanning

'Wash out the flow cell
SyringePump Flowrate (microliter/sec) 100
SyringePump Empty
SyringePump Delay Until Done

'End loop
Loop End

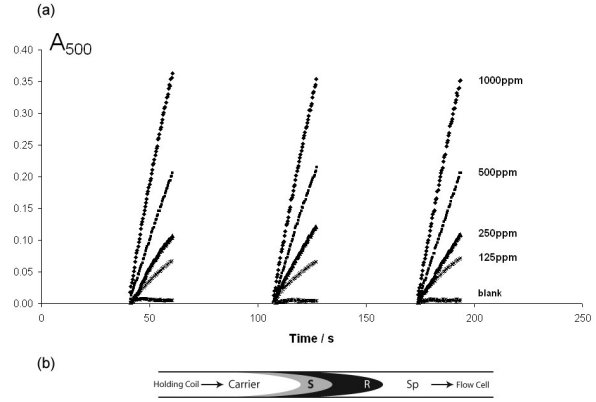

Figure 4: Stopped-flow assay of glucose. (a) The assay of glucose was performed in the range of 125-1000 ppm with 3 replicate runs for each concentration shown superimposed. Data collection was performed only during the stopped flow period, using a dispensed volume of 210 µL to deliver the zones to the flow cell. (b) The stacked zone consisted of 30 µL sample (S), 80 µL reagent (R) and 100 µL spacer (Sp).

A calibration curve was obtained using standard solutions ranging from 125 ppm to 1000 ppm obtained by serial dilution of a 2000 ppm stock solution. The calibration of reaction rate to glucose concentration is linear to 1000 ppm (rate = 1.70 x 10^{-5} [glucose] + 1.01*10^{-3} with R^2 = 0.987) and has a limit of detection of 15.7 ppm. In a teaching laboratory, after performing the calibration, students can assay a number of samples to determine their glucose concentrations.

2.4. Automated Optimization of Assay Protocol.

The microSI-LOV technique allows for automated optimization of experimental variables, such as the volume of injected sample and reagent volumes, as well as the position of the reacting zone within the flow cell, as defined by the volume of flow reversal [9]. The volumes and flow rates shown in the example protocols (Tables 1 and 2) were optimized using an automated protocol (Table 3).

Table 3 Sample protocol for optimization of dispensed volume

Note: Changes to the original phosphate µSI-LOV assay protocol are displayed in bold

Hardware Settings Wavelength 1 (nm) 620

Spectrometer Reference Scan
Spectrometer Absorbance Scanning

'STEP 1: Create new variable
Variable Define New vol
'Assign an initial value
vol = 70

'STEP 4: 6 loops for scanning 70 – 120 µL
Loop Start (#) **6**

'Start-up
SyringePump Flowrate (microliter/sec) 100
SyringePump Valve In
SyringePump Fill
SyringePump Delay Until Done

'Pre-wash
Valve port 2
SyringePump Valve Out
SyringePump Dispense (microliter) 150
SyringePump Delay Until Done

'Aspirate sample
Valve port 5
SyringePump Flowrate (microliter/sec) 10
SyringePump Aspirate (microliter) 10
SyringePump Delay Until Done

'Aspirate reagent 1
Valve port 4
SyringePump Flowrate (microliter/sec) 50
SyringePump Aspirate (microliter) 40
SyringePump Delay Until Done

'Aspirate reagent 2
Valve port 3
SyringePump Aspirate (microliter) 60
SyringePump Delay Until Done

'Send desired section to the flow cell
Spectrometer Reference Scan
Valve port 2
SyringePump Flowrate (microliter/sec) 75
'STEP 2: Replace number with variable name
SyringePump Dispense (microliter) **vol**
SyringePump Delay Until Done

'Stopped flow
Delay (sec) 13

'Wash out the flow cell
Valve port 2
SyringePump Empty
SyringePump Delay Until Done

'STEP 3: Increment variable
vol += 10

'End loop
Loop End

'Shut off spectrometer after last loop
Spectrometer Stop Scanning

3. Conclusion

While FIA will undoubtedly remain widely used, due to the simplicity of its experimental setup, microSI in the lab-on-valve should be considered as a suitable alternative technique, when limited space, robustness, reagent economy, and minimizing chemical waste are of importance. While it has sometimes been inferred that SI inherently offers lower sampling frequency than FI, it is really not the case when performing reaction rate measurements. While the assays presented above have been run at a sampling frequency of one sample per minute, this sampling rate can actually be doubled by loading the second sample and reagent into the holding coil while the first sample is being monitored in the flow cell [10]. Another reason why this paper has focused on stopped flow techniques is that measuring the reaction rate after the flow has been stopped eliminates interfering effects of background color of the reagent or sample, as well as refractive index effects.

In a broader context, microSI-LOV brings flow-based assays into a higher technological level, allowing complex assay protocols to be fully computer controlled and automated with ease. In addition, due to the versatility of flow programming, novel techniques can be conceived including previously impossible operations, such as automatic renewal of column stationary phase, on column detection and many other approaches not yet discovered that will benefit from well-controlled microfluidic manipulations.

Acknowledgments

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