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Redesigning flow injection after 40 years of development: Flow programming

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ABSTRACT

Automation of reagent based assays, by means of Flow Injection (FI), is based on sample processing, in which a sample flows continuously towards and through a detector for quantification of the target analyte. The Achilles heel of this methodology, the legacy of Auto Analyzer®, is continuous reagent consumption, and continuous generation of chemical waste. However, flow programming, assisted by recent advances in precise pumping, combined with the lab-on-valve technique, allows the FI manifold to be designed around a single confluence point through which sample and reagents are sequentially directed by means of a series of flow reversals. This approach results in sample/reagent mixing analogous to the traditional FI, reduces sample and reagent consumption, and uses the stop flow technique for enhancement of the yield of chemical reactions. The feasibility of programmable Flow Injection (pFI) is documented by example of commonly used spectrophotometric assays of, phosphate, nitrate, nitrite and glucose. Experimental details and additional information are available in online tutorial http://www.flowinjectiontutorial.com/

1. Introduction

The individual steps of any assay protocol, i.e., sample and reagent metering, mixing, incubation, monitoring and instrument washout are carried most efficiently at *different time frames and flowrates*. Therefore processing samples by means of constant continuous flow compromises the efficiency of an assay, as much as it would impair driving of a car, equipped with only one forward gear. Yet, the majority of flow based assays including the conventional Flow Injection [1] are still performed on a continuous flow basis, the legacy of Skeggs' [2,3] Auto Analyzer®. The exception is Sequential Injection [4], which uses bidirectional and stop flow to process sample and reagents on intermittent, programmable flow.

There are, however, two limitations of SI methodology; the volume of injected sample cannot exceed 100 microliters, and SI can handle only single or two reagent assays, because in a single line system long multiple zones will not sufficiently overlap and mix. These restrictions do not apply to the Flow Injection technique, since mixing of sample with reagent takes place at the confluence point (red circle, Fig. 1A), where reagent is merged at a steady flow rate along the entire length of sample zone, and where multiple reagents can be added via several confluence points arranged in series between injector and detector. Processing large sample volumes is desirable for trace nutrients assays, where enhanced sensitivity is obtained through analyte preconcentration, or by filling the internal volume of long light path flow cells.

The obvious solution is to combine features of SI and FI methodologies by integrating the injection port with the confluence point within an LOV manifold. (Fig. 1 B, red circle). This manifold configuration, allows sample to be merged by reagent in confluence fashion when the two pumps connected to it operate at different flow rates and directions (Figs. 2 and 11). This novel flow configuration removes limitations of the SI technique and enhances performance of Flow Injection beyond its present capabilities limited by continuous flow [8]. However, the concept of programmable flow injection, pFI, became feasible only recently, when precise pumping by miliGAT pumps was combined with microfluidics within lab-on-valve, and with FLOpro software in a commercially available instrumentation [9].

Here, we describe the operational options and examples for implementing flow programming.

2. Implementation of flow programming

2.1. Programmable flow injection (pFI)

By using a lab-on-valve platform to accommodate the microfluidic manipulations, the miniaturized pFI technique is based on synchronized merging of sample zone with reagent zones, at a confluence point situated at the central port of the LOV manifold (Fig. 1B). In distinction to the established continuous FI mode of operation (cFI), the sample zone is in the pFI format, mixed with reagents *upstream from the*

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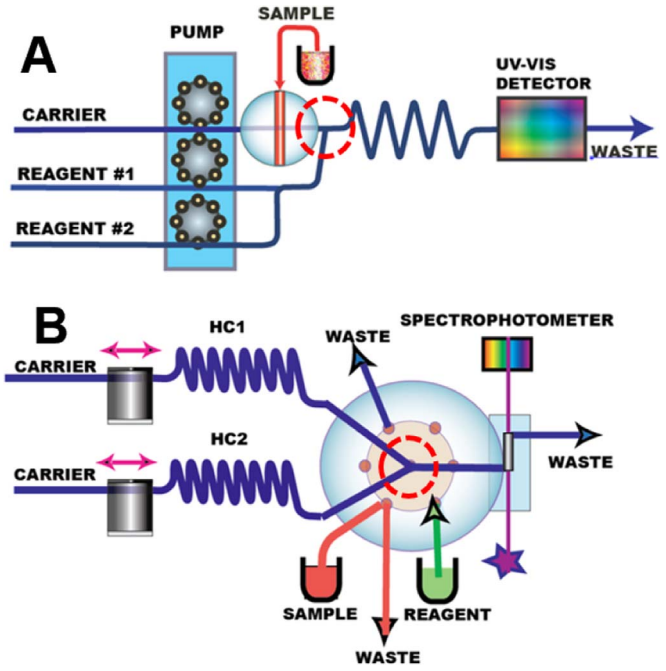


Fig. 1. A. Flow injection manifold. B. Programmable flow injection in lab-on-valve format. Red circle indicates confluence point (from Ref. [7]). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

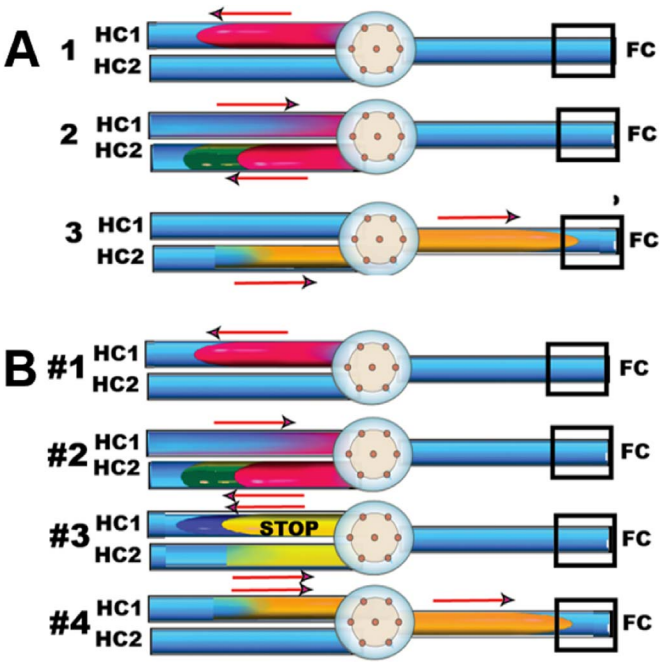


Fig. 2. Steps of programmable flow injection protocol. A. Single reagent assay. B. Two reagent assay. (From Ref. [7]).

confluence point, inside holding coils (HC1,HC2). In this way sample zone is supplied along its entire length with an even concentration of reagent(s) – as in traditional FI. Moreover, sample also experiences *flow reversals* before reaching the flow through cell. In addition, the flow programming also allows insertion of a stop flow period anywhere on the flow path, thus providing time for incubation of sample with reagents.

The assay protocol for a *single reagent* method (Fig. 2A) comprises the following steps:

1) Sample (red) is aspirated into holding coil (HC1)
2) Sample is transferred into HC2 simultaneously with reagent (green), added at the confluence point. The mixing ratio of sample with reagent is controlled by combining the delivery rate of pump #1 with the aspiration rate of pump #2
3) The reaction product (orange) is moved through the flow cell for monitoring by forward flow generated by pump #2.

The assay protocol for a two reagent method (Fig. 2B) is an extension of the single reagent protocol, by addition of the second reagent in step #3. In this protocol the sample zone experiences three flow reversals.

The injected sample volume, as well as volumes of reagents and their mixing ratios are controlled by software protocol. Therefore the same flow system will accommodate a wide variety of reagent based assays, without need for physical reconfiguration, such as of the volume of sample injection loop, or of the length of reaction coil, or of change of internal diameter of peristaltic pump tubing.

Stopping the flow is a versatile approach for optimizing sensitivity of an assay, because it allows inclusion of an incubation period for sample with reagents. The reaction mixture can be arrested either in the holding coil (SHC method), or in a flow cell (SFC method). Depending on the requirements of an assay, the holding coil can be thermostated, and the stop flow executed at a selected stage of a protocol.

2.2. Stopping flow in the holding coil and flow programming

Incubation of sample with reagents by stopping the flow in the *holding coil* (Fig. 3A) yields readout analogous to a flow injection peak (Fig. 3B). The height of such peak, recorded while sample zone flows through the detector, is proportional to the concentration of the target analyte. Arresting the reaction mixture in the holding coil offers the opportunity to control the temperature of incubation, and in this manner the reaction rate and sensitivity of the assay.

While duration of the stop flow period applied to incubation impacts sensitivity of the assay, programming of flow rates applied to

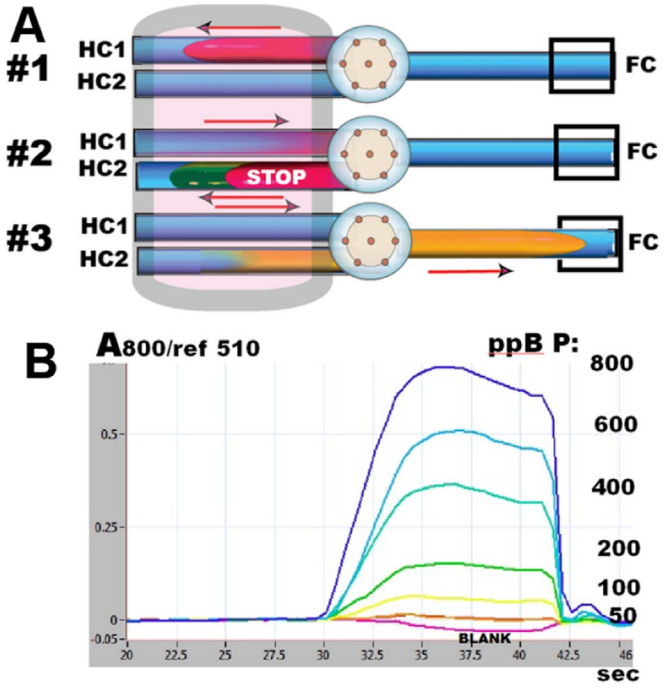


Fig. 3. The SHC protocol. A. Sequence of steps for single reagent assay, with single sample transfer. B. Response curves shown superimposed experimental runs of phosphate assay. (From Ref. [7]).

individual assay steps allows optimization of sampling frequency and insures washout of the flow channel, with the aim to avoid carryover. Typically a pFI assay cycle comprises the following steps: 1/ washout with carrier for 2 s at 250 µL/s, 2/ set up baseline and begin data collection,

3/ aspiration of sample for 2 s at 50 µL/s, 4/transfer sample from HC1 to HC2 for 2 s at 50 µL/s, while reagent is confluenced at 25 µL/s into HC2, 4/ stop flow incubation period of 5 s in HC2,

5/ transport of reacted sample zone through flow cell at 25 µL/s, 6/ washout with carrier for 2 s at 250 µL/s, 7/stop data collection. Detailed protocols for assay of nitrate [10], nitrite [11], and phosphate [12], illustrate the versatility of flow programming. Since the majority of these assays use deionized water as carrier, and because volumes consumed and generated per assay are far smaller compared to the traditional FI, flow programming is a tool that supports the concept of green chemistry.

2.3. Examples of nutrient assays in SHC mode

With the aim to illustrate the versatility of the pFI SHC technique, two assays are briefly discussed in this section; the simple single reagent assay of *nitrite* and the assay of *nitrate* that involves complex microfluidic manipulations.

The *nitrite assay* is based on the Gries reaction, whereby nitrite is converted into red azo dye monitored at 500 nm, using a flow cell with 13 cm long light path and stop flow period of 5 s in the holding coil thermostated at 25 C. The software script defines sample volume of 100 µL. DI water was used as carrier, and composition of reagent and detailed software script is in [11].

Calibration run (Fig. 4A), shows six superimposed response curves, comprising initial baseline, a dip, due to refractive index between DI

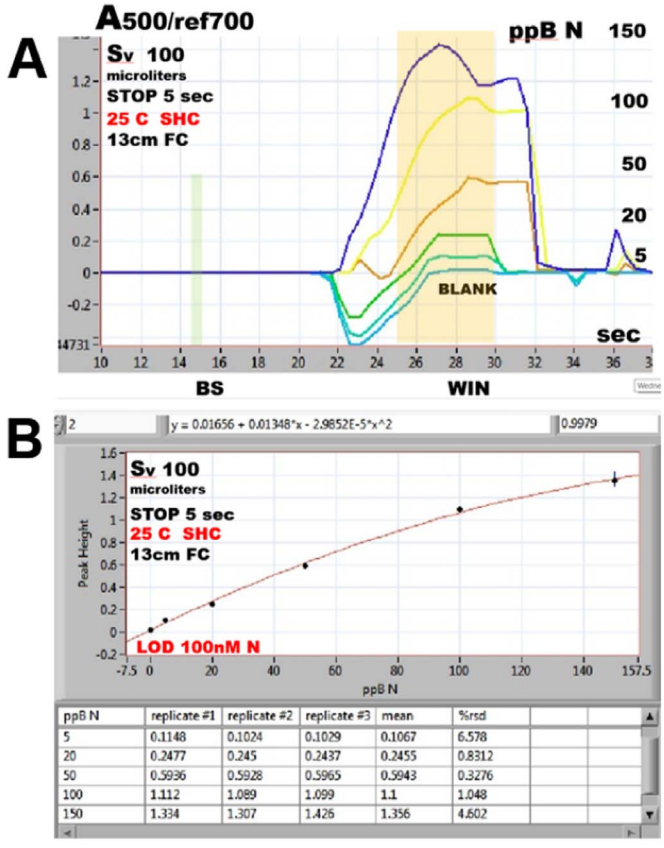


Fig. 4. Assay of nitrite using pFI SHC protocol. Superimposed response curves of the calibration experiment. B. Calibration graph and tabulated reproducibility data. (From Ref. [7]).

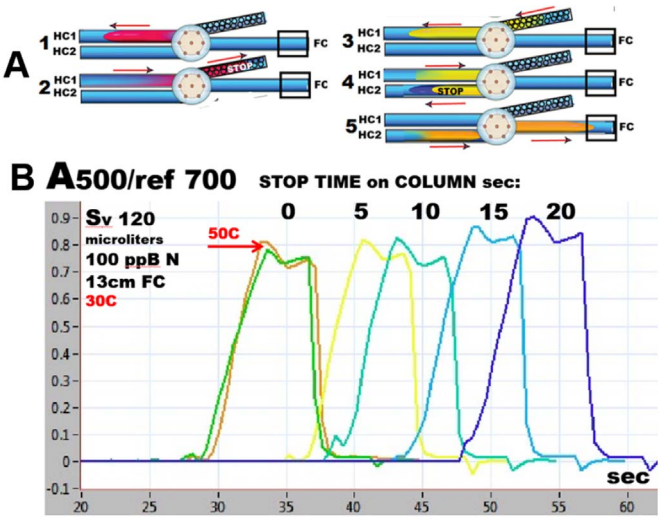


Fig. 5. Assay of nitrate using pFI SHC protocol. A. Sequence of steps for on column stop assay with three sample transfers. B. Kinetics of on column reduction for increasing incubation times and elevated temperature. (from Ref. [7]). (For interpretation of the references to color in this figure, the reader is referred to the web version of this article).

and reagent, and peak shaped responses of increasing height.

The calibration graph (Fig. 4B) was obtained by automated data collection and by plotting the difference of absorbance values between baseline (BS) and peak maximum located within the window (WIN). This single transfer SHC method has an average r.s.d. of 2.7%.

Nitrate assay is based on reduction of nitrate to nitrite on a cadmium column, followed by conversion of nitrite, by the Gries reaction, into red azo dye monitored at 500 nm.

Therefore automation of nitrate assay in the pFI mode requires the following sequence of microfluidic manipulations (Fig. 5A):

1) Sample (red) is aspirated into HC1,
2) Sample is transferred to Cd column,
3) Resulting nitrite (yellow) is transferred back into HC1,
4) Gries reagent (green) is aspirated into HC2, simultaneously with nitrite transferred from HC2,
5) The resulting azo dye (orange) is flowed through the flow cell for monitoring.

The reaction rates of nitrate reduction and of dye formation influence the sensitivity of this assay, and its sampling frequency. The Gries reaction is very fast and therefore the assay protocol does not need to include a stop period in step #4. Kinetics of on column reduction depends on column geometry, volume and surface area of the reductor. In a 10 cm long column, reduction proceeds very fast, as revealed by the experiment where sample was held on column for increasing periods of time (Fig. 5B). Elevating the temperature of the flow system from 30 °C to 50 °C (red arrow), did not affect the reaction yield, as this was, surprisingly, already close to 100% at 0 s long stop time on column held at 30 °C. Ammonium chloride buffer was used as carrier in order to keep the Cd column active. Reagent composition, column reactivation and detailed assay protocol are in [10].

In order to maximize sensitivity of this assay, the injected sample volume was increased to 120 µL and sample was held on column for 15 s. The calibration experiments were performed by analyzing a series of nitrate standards, in the range of 0–200 ppB N, by means of the assay protocol outlined above. The response curves, shown superimposed (Fig. 6A) were evaluated by collecting peak height measurement in a window (WIN) and subtracting baseline data from BS interval. The resulting calibration curve (Fig. 6B), indicates an LOD of 33 nM of nitrate nitrogen and average r.s.d. 3.3%. These values are better or comparable with data obtained by the traditional continuous

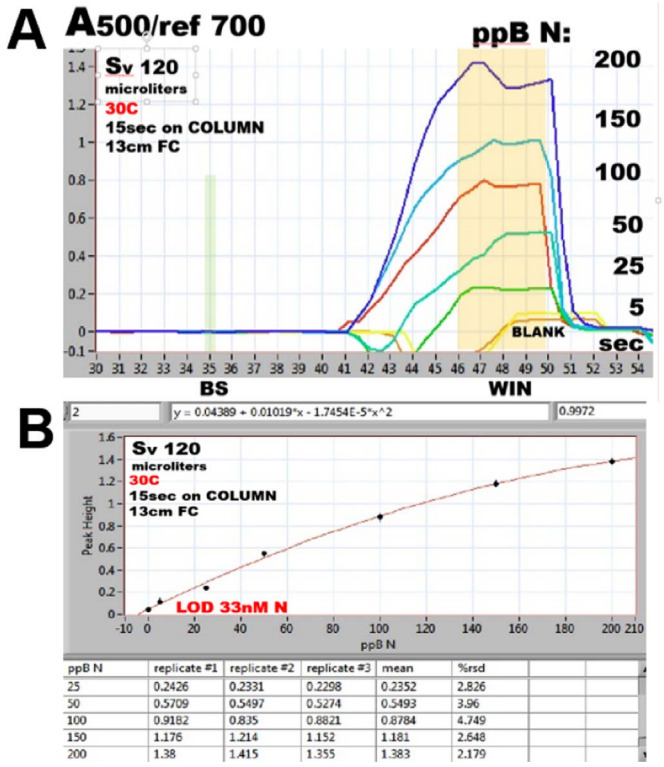


Fig. 6. Assay of nitrate using pFI SHC protocol. Superimposed response curves of the calibration experiment. B. Calibration graph and tabulated reproducibility data. (From Ref. [7]).

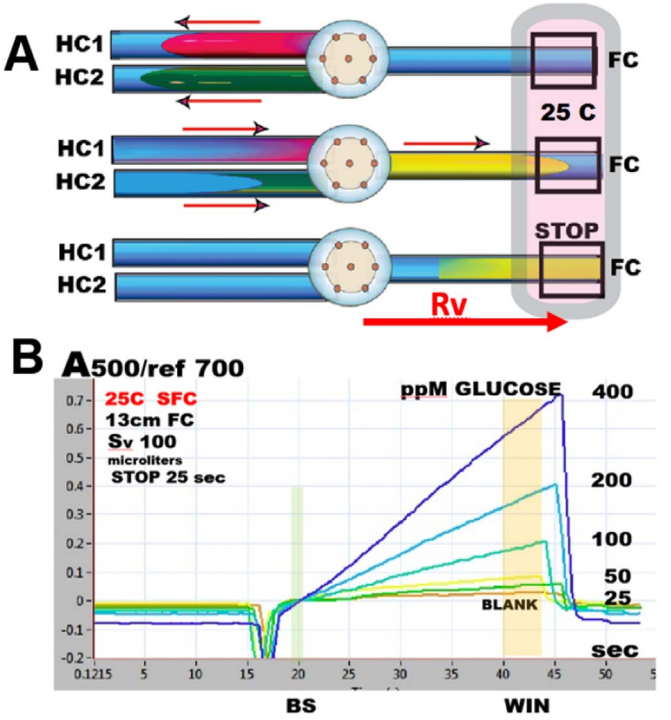


Fig. 7. The SFC protocol. A. Sequence of steps for single reagent assay, without sample transfer. B. Reaction rate curves of glucose are shown superimposed (from Ref. [7]). (For interpretation of the references to color in this figure, the reader is referred to the web version of this article)..

flow techniques, where on column reduction is known to be the source of larger errors.

Programmable flow injection in the SHC mode was also applied to two reagent assay of phosphate, based of spectrophotometric measurement of phosphomolybdenum blue, where the sample zone experiences two transfers between the holding coils [12]. The calibration graph was linear within the range of 0 to 800ppB P with average r.s.d 4.4% and LOD 500 nM.

2.4. *Flow programming in SFC mode and assay of glucose and nitrite*

The single reagent SFC protocol (Fig. 7A), comprises the following steps:

1) Sample (red) is aspirated into HC1,
2) Reagent (green) is aspirated into HC2
3) Sample and reagent are simultaneously propelled through the confluence point towards the detector,
4) The resulting product (orange) is arrested in the flow cell for reaction rate monitoring. The responses (Fig. 7B) are reaction rate curves, the slope of which is proportional to the concentration of the target analyte. The temperature of the reacting mixture can be elevated, in order to accelerate reaction rate [13].

Because the SFC protocol is based on selection of a *portion* of dispersed sample zone, it has to be optimized by selecting a *return volume* (Rv Fig. 7A), designed to place the centroid of the sample zone in the middle of the 13 cm long light path of the flow cell [14].

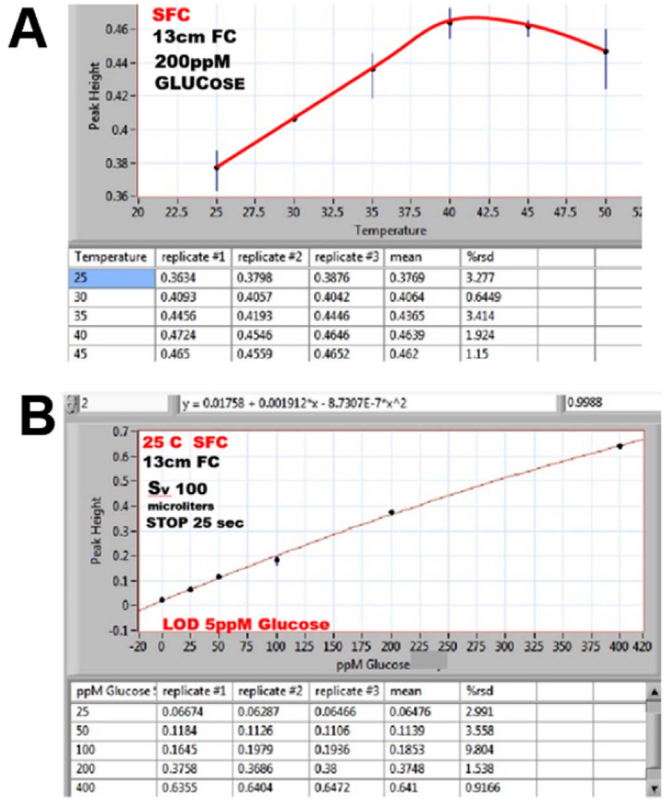
Assays performed in the SFC mode can be based either on the *end point* measurement, or on the *slope* of the reaction rate response. For fast chemical reactions, the end point measurement is the only option, while slower reaction kinetics can be evaluated by either method. The kinetic assay of glucose and of nitrite illustrate these alternatives.

Assay of glucose (Fig. 7) is based on a two-step reaction, producing red quinoeminine dye monitored at 500 nm. The reactants, available in a single solution reagent kit, were used along with DI water as a carrier. Further details as well as software protocol are available in [15].

When optimizing the *glucose* assay, the influence of temperature was investigated first by using 100 μL of 200 ppm glucose standard and by measuring peak height while changing temperature of the flow cell from 25 to 50 °C for an incubation period of 25 s (Fig. 8A). Since little is gained in terms of peak height and sensitivity of assay by elevating temperature, 25 °C was used for calibration experiments in order to ensure adequate thermostating of the flow cell (above ambient 21 °C).

Reaction rate curves (Fig. 7B) recorded during 25 s long stop flow period show increasing slope, with the concentration of glucose, while in absence of glucose (BLANK) there is no increase of absorbance. The calibration graph (Fig. 8B), based on *slope* of reaction rate curve was obtained by subtracting A-value at SB time, from A-value at WIN time. Reproducibility of all triplicate measurements shows average of 3.8% r.s.d.

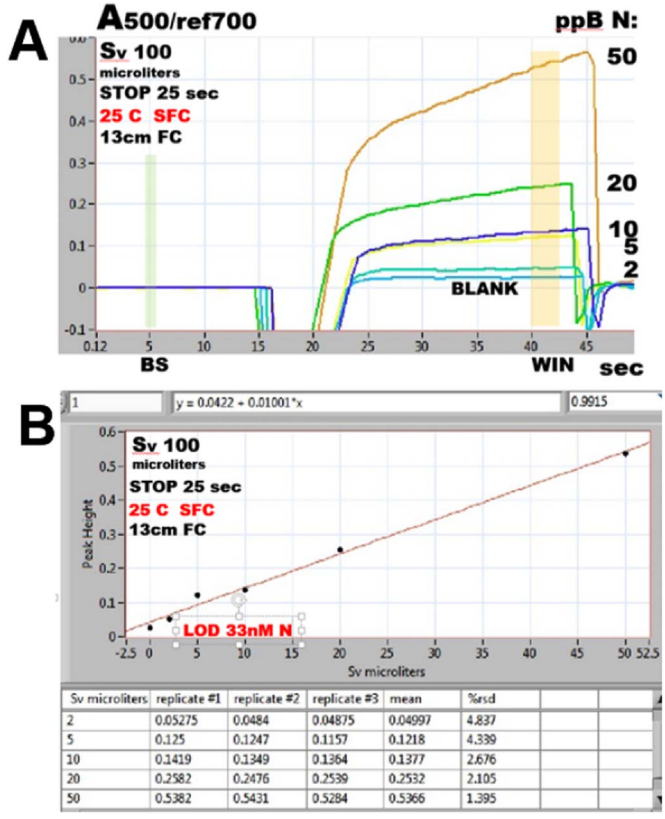
The colorimetric assay of *nitrite* discussed in Section 3, in the SHC format, is performed here in the SFC format, in order to illustrate how very fast reaction kinetics, such as of the Gries reaction, can be monitored to yield reproducible results. The assay protocol, designed to bring sample and reagent into a flow cell rapidly, with the aim to maximize detector response, by capturing chemical reactions from the very beginning, is the same as the one used for glucose assay (Fig. 7A), but uses *end point*, instead of reaction rate measurement. By using a stop flow period of 25 s for sample incubation in a 13 cm long flow cell at temperature of 25 °C, a series on nitrite standards was analyzed and the thus obtained response curves were superimposed (Fig. 9A). The difference of A values between baseline (BS) and at the end of the stop flow period (WIN) yields a linear calibration with LOD of 33 nM N. and average r.s.d. of 3.1%. Note that an attempt to use reaction rate measurement, as in the case of glucose, by placing SB at 20 s (Fig. 7B) would yield irreproducible results. Additional information on nitrite SFC assay is available at [16].



Temperature	replicate #1	replicate #2	replicate #3	mean	%rsd
25	0.3634	0.3798	0.3876	0.3769	3.277
30	0.4093	0.4057	0.4042	0.4064	0.6449
35	0.4456	0.4193	0.4446	0.4365	3.414
40	0.4724	0.4546	0.4646	0.4639	1.924
45	0.465	0.4559	0.4652	0.462	1.15

ppM Glucose	replicate #1	replicate #2	replicate #3	mean	%rsd
25	0.06674	0.06287	0.06466	0.06476	2.991
50	0.1184	0.1126	0.1106	0.1139	3.558
100	0.1645	0.1979	0.1936	0.1853	9.804
200	0.3758	0.3686	0.38	0.3748	1.538
400	0.6355	0.6404	0.6472	0.641	0.9166

Fig. 8. Reaction rate based assay of glucose in SFC format. A. Influence of temperature on sensitivity. B. Calibration graph of glucose assay and tabulated reproducibility data. (From Ref. [7]).



Sv microliters	replicate #1	replicate #2	replicate #3	mean	%rsd
2	0.05275	0.0484	0.04875	0.04997	4.837
5	0.125	0.1247	0.1157	0.1218	4.339
10	0.1419	0.1349	0.1364	0.1377	2.676
20	0.2582	0.2476	0.2539	0.2532	2.105
50	0.5382	0.5431	0.5284	0.5366	1.395

Fig. 9. End point based assay of nitrite in SFC format. A. Superimposed reaction rate curves of the calibration experiment. B. Calibration graph and tabulated reproducibility data. (From Ref. [7]).

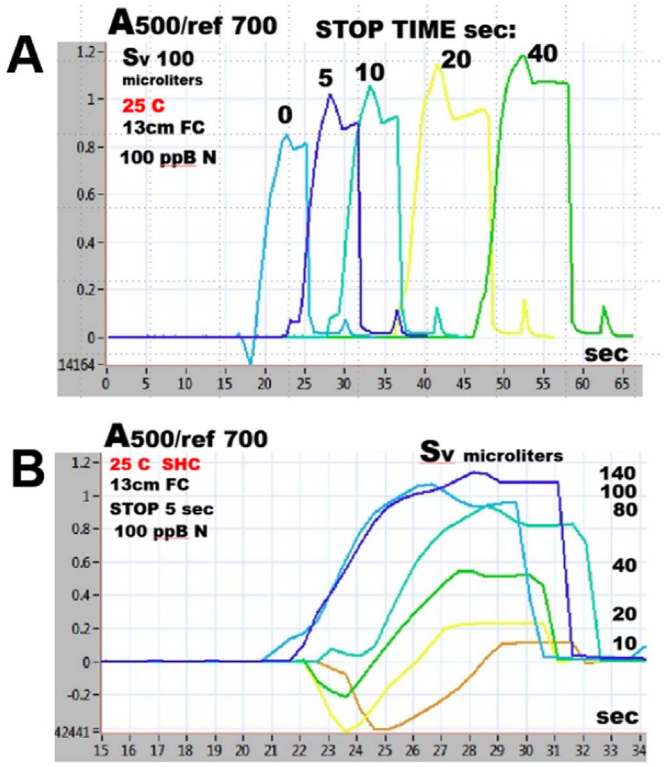


Fig. 10. Nitrite assay in SHC mode. A. Influence of incubation time on sensitivity. B. Influence of processed sample volume on sensitivity. (From Ref. [7]).

2.5. Tools for optimization of pFI based assays

Performance of the continuous flow and of the programmable Flow Injection techniques is optimized by using the same tools, but the difference is that for cFI the flow system has to be physically reconfigured, while for pFI the same result is achieved by changing the software protocol without need for physical reconfiguration of the manifold components.

The *incubation period* in cFI is adjusted either by changing the length of the mixing coil, or by slowing the flow rate by changing tubing in the peristaltic pump. In the pFI SHC format it is the duration of the stop flow period (Fig. 10A), adjusted by software script. The example shown here is the series of superimposed response curves, of nitrite analysis, recorded with incubation periods of 0–40 s. Based on this experiment, the incubation period in the optimized assay protocol was set to 5 s (Fig. 4) [11].

The *concentration range and sensitivity* of an assay in cFI format is adjusted by changing the volume of the injection loop mounted on the injection valve. In the pFI format this is achieved by programming the volume (Sv) of sample aspirated by pump #1 into holding coil #1 (Fig. 3A step #1), [11]. The increase in peak height with increase of Sv (Fig. 10B) follows the same asymptotic pattern, established for the traditional continuous flow injection [17].

The *mixing ratio of sample with reagent* at the confluence point in cFI format is adjusted by changing the internal diameters of tubing delivering carrier solution and reagent solution to the confluence point. Since this is a laborious task, optimization of this parameter is seldom done in a systematic way. In pFI format (Fig. 11A), the mixing ratio of sample with reagent is adjusted by programming the flowrate of the pump P1 delivering sample zone (red) to the confluence point, to be lower than the flow rate of P2 aspirating the reaction mixture into holding coil #2.

The flow rates shown in Fig. 11A yield a sample reagent ratio 5:1. This ratio yields tall and narrow peaks (Fig. 11B top curve). Increasing flow rates of reagent, while the sample flow rate is held the same, yields

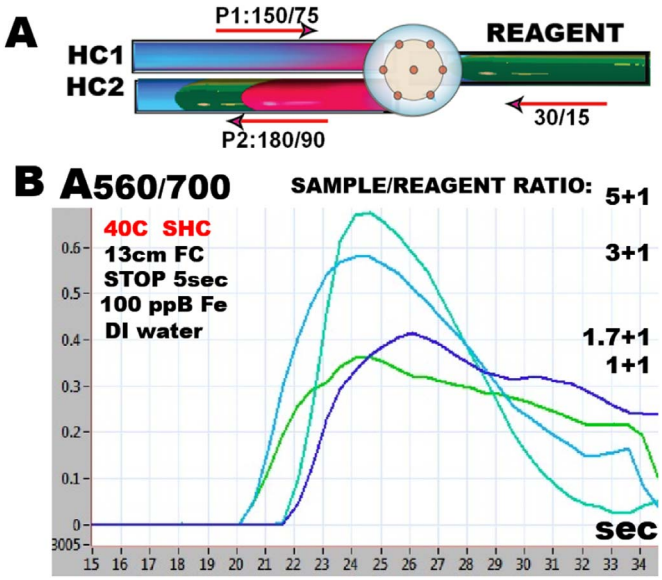


Fig. 11. Mixing of sample with reagent at confluence point. Assay of iron II in SHC, single reagent, single transfer mode. A. Combined flow rates of sample and reagent yield mixing ratio 5:1. B. Response curves obtained in SHC mode for various mixing ratios. (From Ref. [7]). (For interpretation of the references to color in this figure, the reader is referred to the web version of this article)..

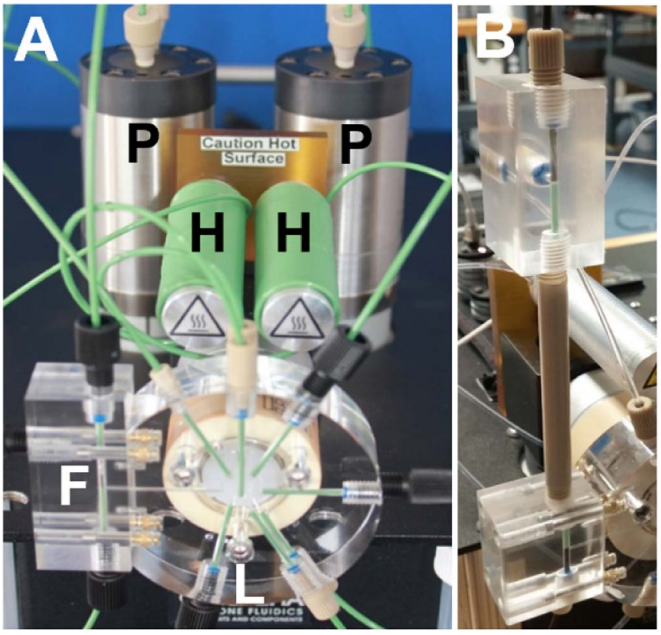


Fig. 12. miniSIA-2 instrument. A. Two miliGAT pumps (P), two holding coils (H), lab-on-valve module (L), flow cell (F). B. Flow cell with `13 cm long light path.

lower and broader responses as the sample zone becomes progressively more diluted. This example, of iron (II), of spectrophotometric assay by ferrozine, shows that 150 μL of sample can be best analyzed using only 30 μL of reagent, demonstrating how economical is pFI in terms of reagent consumption. Application of this methodology for analysis of sea water has recently been developed [5].

Optimizing the automated method by means of software programming rather than by physical reconfiguration of the manifold, is not only convenient, but also more reliable, since it yields a better defined assay protocol. The result is that a method developed and defined by software script can be reproducibly repeated on other instruments and locations.

2.6. Instrument configurations

The instrument used in this work (Fig. 12), is commercially available [9]. It comprises two miliGAT pumps (P), two thermostated holding coils (H), a six position lab-on-valve module (F), flow cell (F), spectrophotometer and Xenon light source. The flow cell, mounted externally on a lab-on-valve module, was in this work replaced by the 13 cm long light path flow cell (Fig. 12B), with the aim to increase the sensitivity of spectrophotometric measurements. The miliGAT has a unique construction that combines advantages of peristaltic pumping, the continuous refilling, with advantages of a syringe pump. It provides a wide range of flow rates, in forward and reverse direction, and is computer compatible. Its unique construction is based on four micro syringes that are moved around on a slanted carousel, the speed of which controls the flowrate, while the degree of rotation controls the delivered volume [18].

3. Conclusions

Redesigning the flow injection manifold, by integrating sample injection with confluence into a single point within the lab-on- valve module, results in a multipurpose flow system that accommodates a variety of reagent based assays without the need for physical reconfiguration. On this platform, flow programming lends an unprecedented ease to optimization of assay protocols, and to exploration of reaction kinetics of chemical reactions. While almost trivial, the idea of programmable Flow Injection would not have been feasible without advances in precision bidirectional micro pumping that makes complex microfluidic manipulations reproducible. It was therefore essential to perform well known reagent based assays in the two formats, SHC and SFC, in order to establish reproducibility of pFI, which was found to be in an average r.s.d. of 3.1% This level of precision is in agreement with precision of spectrophotometry [6], used here as the detection technique. Also, application of pFI to nutrient assays resulted in equal or better sensitivity and limit of detection, as compared to the continuous flow techniques.

It would be unrealistic to expect that pFI, due to its indisputable advantages, will replace cFI any time soon. Continuous flow techniques, including air segmented methods, are well entrenched, and will remain in use, and rightly so, since they are widely used, are transparent to users, and are supported by instrument manufacturers. The existing inventory and production of these “legacy techniques” will continue to serve well to routine users in daily tasks of performing serial assays, also because cFI offers slightly higher sampling frequency than pFI. It remains to be seen if lower reagent consumption and waste generation, desirable from viewpoint of green chemistry, combined with small footprint of the instrument, will outweigh this single drawback of pFI in time. Also, now, when peristaltic pumping is finally eliminated from Flow Injection methodology, its drawbacks, including the need to replacement peristaltic tubing, becomes the thing of the past.

The main purpose of this work is, however, to emphasize versatility and advantages of flow programming, and to inspire research in this new avenue, opened by recent advances in precision pumping, by microfluidics in lab-on-valve format and by associated instrumentation.

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