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Programmable Flow Injection. Principle, methodology and application for trace analysis of iron in a sea water matrix

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ABSTRACT

Automation of reagent based assays by Flow Injection is based on sample processing, in which a sample flows continuously towards and through a detector for monitoring of its components. There are three drawbacks to using this approach. The constant *continuous forward flow*: continually consumes reagents and generates chemical waste and necessitates a compromise when optimizing the performance of the reagent based assay. The reason is that individual steps of an assay protocol, i.e., sample and reagent metering, mixing, incubation, monitoring and efficient washout are carried out most efficiently on different time scales and therefore at different flowrates. Programmable Flow Injection (pFI) eliminates all three drawbacks and permits the execution of optimization of the assay protocol by means of a computer. This paper details this novel approach to method development by optimization of an assay of iron at nanomolar levels and its application to its determination in a sea water matrix. The pFI method was developed in two variants: Stop in Holding Coil (SHC) and Stop in Flow cell (SFC). The SHC method has a Limit of Detection (LOD = 3.1 ppb or 55 nM Fe, precision of 1.9% r.s.d. at ~ 90 nM, and sampling frequency of 90 samples/h. The SFC method had LOD = 0.57 ppb or 10 nM Fe, precision of 0.8% r.s.d. at ~ 90 nM, and sampling frequency of 40 samples/h and its sensitivity is independent of the salinity of the matrix. The SFC method, and its manual equivalent, was used for the determination of dissolved Fe (II) that had been spiked into several samples of seawater that had been diluted with various volumes of deionized water to mimic coastal seawater. The results showed good agreement between both the SFC and the manual methods.

1. Introduction and principles

Automation of reagent based assays that are based on a *continuous flow* format is difficult to optimize, since the various steps of the assay protocol, i.e., sample and reagent metering, mixing, incubation, monitoring and instrument washout, are best performed at different flowrates. Also, processing samples by means of an uninterrupted flow continuously consumes reagents and generates chemical waste. Yet, in spite of these drawbacks, the majority of flow based assays, including Flow Injection [1,12] are still performed on a continuous flow basis, the legacy of Skeggs' [2,3] Auto Analyzer®.

Flow programming, recently applied to Flow Injection [4,13], avoids these shortcomings by utilizing stops and reversed and accelerated flowrates that are tailored to the needs of the individual steps of the assay protocol. The key feature of the miniaturized programmable Flow Injection (pFI) system is the integration of the sample injection with a confluence point at the central port of the lab-on-valve (LOV) manifold (Fig. 1, red circle). The key components of the instrument [14] are the two high precision bidirectional pumps, connected to the LOV manifold via two thermostated holding coils. Monitoring takes place in an externally mounted, long light path flow cell, interfaced with a spectrophotometer and light source by fiber optic cables.

The assay protocol, controlled by a software script, has two basic modes of operation:

- The Stop in Holding Coil (SHC) method is based on the incubation of the sample/reagent mixture in the holding coil.
- The Stop in Flow cell (SFC) method is based on the incubation of the sample/reagent mixture in the flow cell.

For single reagent assays the SHC protocol (Fig. 2A) comprises the following steps:

1) The sample (red) is aspirated by pump#1 (P1) into the holding coil (HC1).
2) The sample is transferred into HC2 simultaneously with a reagent (green), which is added at the confluence point. The mixing ratio of the sample with the reagent is controlled by combining the delivery

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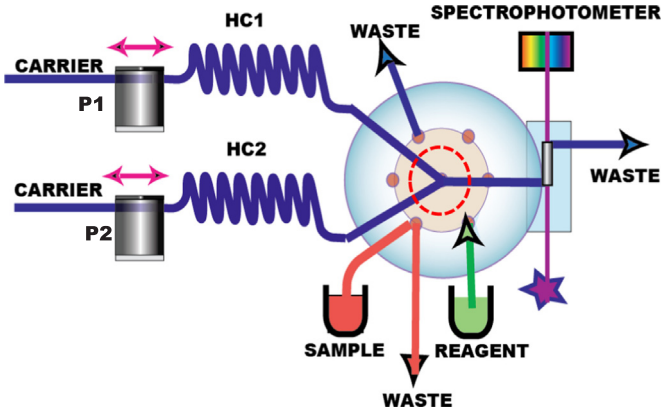

Fig. 1. Schematic of the programmable Flow Injection instrument containing a Lab on Valve (LOV) and milliGAT pumps P1. & P2. and holding coils HC1 & HC2. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

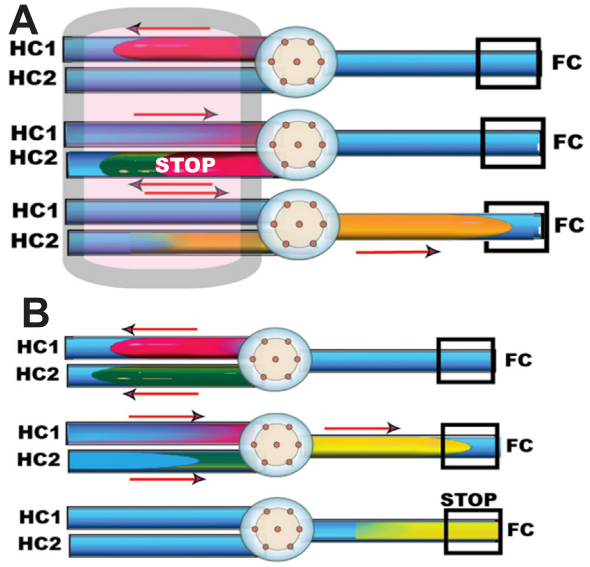

Fig. 2. Schematic of the sequence of the assay protocol of programmable Flow Injection methods. A. Stopped in holding coil method (SHC). B. Stopped in flow cell method (SFC). (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

rate of P1 with the aspiration rate of pump #2 (P2).
3) The reaction mixture is held in HC2 for incubation.
4) The reaction product (orange) is moved through the flow cell for monitoring by a forward flow generated by P2.

The response curves have the shape of a peak analogous to that of traditional Flow Injection (Fig. 7), the height of the peak is proportional to concentration of the target analyte.

For single reagent assays the SFC protocol (Fig. 2B) comprises the following steps;

1) A sample (red) is aspirated by P1 into HC1.
2) A reagent (green) is aspirated by P2 into HC2.
3) The sample and reagent are then simultaneously propelled through the confluence point towards the flow cell.
4) The resulting product (yellow) is held in the flow cell and the reaction rate is monitored.

The responses have the form of reaction rate curves, the slope of which is proportional to the concentration of the target analyte [16]. However, for very fast chemical reactions, such as the formation of the iron (II) ferrozine® complex, the end point measurement (Fig. 9) is used to construct the calibration graph.

The distribution, bioavailability and speciation of iron in hydrothermal systems and in ocean waters and the mechanisms by which Fe is added to the oceans is of great interest to oceanography as it has a major influence on the biological community structure and its overall primary production. The distribution of Fe in these aquatic environments has been measured by spectrophotometry using several reagents of which ferrozine has a unique capability to measure Fe (II) as well as total Fe concentration. The ferrozine method has been recently automated in FI format and successfully used for the study of hydrothermal vents and other ocean waters [5]. The potential release of Fe (II) from reducing coastal sediments and its role in ameliorating Fe limitation [6] gave us the opportunity to test and compare the performance of programmable flow injection with manually performed assays on samples from this research program.

Ferrozine®, sodium salt of pyridyldiphenyltriazine sulfonic acid (m.w. 492.46) is related to the group of ferroin reagents, that all form water soluble colored chelates with iron (II) and Cu (I). Red ferrozine-Fe (II) chelate has a molar extinction coefficient $\varepsilon = 2.86 \times 10^4$ at 562 nm and is formed in acetate buffer at pH = 4.5. In the presence of the reducing agent hydroxylamine, it can also be used for the assay of total Fe (II + III) content [7]. The ferrozine method has also been used for the analysis of Fe (II) in natural waters after preconcentration of the complex on C-18 [8] with a Limit of Detection (LOD) 0.1 nM. An assay of Fe by automated spectrophotometry without preconcentration by means of 'chip based' instrument in sea water [9] achieved a LOD 27 nM at sampling rate of 12 samples/h. Manual spectrophotometry of the ferrozine-Fe(II) complex using a 4.5 m long flow cell reached a LOD of 0.2 nM Fe with a working range of 0.5–10 nM in sea water [10].

2. Materials and methods

2.1. Instrumentation

The instrument, miniSIA-2 (GlobalFIA, Fox Island, WA, USA), fitted with 13 cm long flow cell, was used without any modifications. The instrument comprises two high precision, synchronously refilling milliGAT pumps, two thermostated holding coils, a 6-port lab-on-valve module with an external flow cell (Fig. 1). All tubing connections, downstream from the milliGAT pumps including the holding coils (volume 1000 µL), were made with 0.8 mm I.D. polytetrafluoroethylene (PTFE). The tubing between the carrier stream reservoirs and the milliGAT pump was made from 1.6 mm I.D. PTFE tubing in order to minimize degassing under reduced pressure at higher aspiration flow-rates. A spectrophotometer (USB4000, Ocean Optics, Dunedin, FL) and a tungsten light source within miniSIA-2 instrument were connected to the flow cell using optical fibers. All assay steps were computer controlled using commercially available software (FloZF, GlobalFIA, Seattle, USA).

2.2. Reagents and materials

0.01 M Ferrozine solution was prepared by dissolving 0.492 g FerroZine™ iron reagent (ACROS Organics, www.acros.com) in 100 mL 0.2 M ammonium acetate buffer. The solution was prepared weekly, and stored refrigerated in the dark.

0.2 M Ammonium acetate buffer (pH 4.6) was prepared from isothermal distilled acetic acid Certified ACS (Fisher Scientific, www.fishersci.com) and ammonium hydroxide, Certified ACS PLUS (Fisher Scientific, www.fishersci.com) in deionized water [11].

Deionized water was prepared using a Barnstead Water Purification System, Nano Pure Diamond (Thermo Fisher Scientific, www.thermofisher.com). It is important that the water is free from excess dissolved gases, this is the best accomplished by storing water over

night before use or by stirring under vacuum.

Hydrochloric acid, Certified ACS PLUS (Fisher Scientific, www.fishersci.com) was purified by isothermal distillation [11].

Sodium chloride (EMD Chemicals Inc., Gibbstown, NJ).

Hydroxylamine hydrochloride, Certified ACS (Fisher Scientific, www.fishersci.com).

Sea water samples were collected from Kewalo Marine Laboratory, and were filtered through a 0.45 μm filter (Supor 450 Membrane filter, Pall, https://shop.pall.com/), and were stored acidified to pH2 using purified HCl.

A 2.00 ppm stock standard iron solution was prepared by diluting a 200 ppm commercial iron standard (LabChem, Zelienople, PA) in 0.01 M HCl and 0.5% hydroxylamine hydrochloride.

The stock solution was further diluted to obtain working standards in 0.01 M HCl, 3.5% NaCl in 0.01 M HCl, and seawater in 0.01 M HCl.

The carrier solution was prepared using 0.2 M ammonium acetate buffer without any surfactant.

Seawater samples of different salinities were prepared by diluting with deionized water and spiking with Fe (II) to mimic coastal seawater (Table 2). Each sample was divided into two portions and determined by both the manual and the SFC methods.

2.3. The manual method

The manual method was performed in batch mode, by mixing the sample with reagent in a ratio (S/R) 4/1. The resulting mixture was automatically aspirated into the miniSIA-2 instrument furnished with 13 cm long light path flow cell for absorbance measurement at 560 nm using 700 nm as a reference. Calibration standards and sea water samples were treated in exactly the same way as follows:

1. Add 2 mL of reagent in 15 mL polymer test tube.
2. Add 4 mL of sample into the test tube.
3. Mix the solution well and wait 3 min.
4. Aspirate 270 μL of reaction mixture into the 13 cm flow cell via the LOV.
5. The absorbance is measured after 20 s.

2.4. Development and optimization of the SHC method

Optimization of the key reaction and data collection parameters is much easier using the SHC method than using the SFC method, [13] and since the optimized SHC protocol (Fig. 3) can easily be modified for the SFC method the optimization process for the SHC method is explained in detail here. For trace analysis work the priority is to achieve maximum sensitivity and the lowest limit of detection of the target

FLOWCELL	COV		port #	2
Pump 1 dispense	Pump 1		volume (μL), flow rate	500,250,0
Pump2 dispense	Pump2		volume (μL), flow rate	500,250,1
Spec get reference spectrun	Spec		N/A	N/A
Spec start acquire	Spec		request period (seconc	0.5
SAMPLE	COV		port #	4
Pump 1 aspirate	Pump 1	Sv	volume (μL), flow rate	300,150,1
REAGENT #1	COV		port #	3
Pump2 aspirate	Pump2	S/R RATIO	volume (μL), flow rate	180,90,0
Pump 1 dispense	Pump 1		volume (μL), flow rate	150,75,1
wait	System	INCUBATE	time (s)	5
FLOWCELL	COV		port #	2
Pump2 dispense	Pump2		volume (μL), flow rate	300,25,1
Pump2 dispense	Pump2		volume (μL), flow rate	500,250,1
Spec stop acquire	Spec		N/A	N/A
Pump 1 dispense	Pump 1		volume (μL), flow rate	500,250,1
DATA				
set data window	Data	WIN	min time (s), max time	27,33,1
subtract baseline	Data		at time (s), data index (	15
calc peak height	Data		data index (optional), f	1
activate table by numbe	Data		table number	84
add to calib table	Data		standard value	50
save data to file	Data		N/A	N/A

Fig. 3. FloZF program steps for the stopped in holding coil (SHC) method. For details see text.

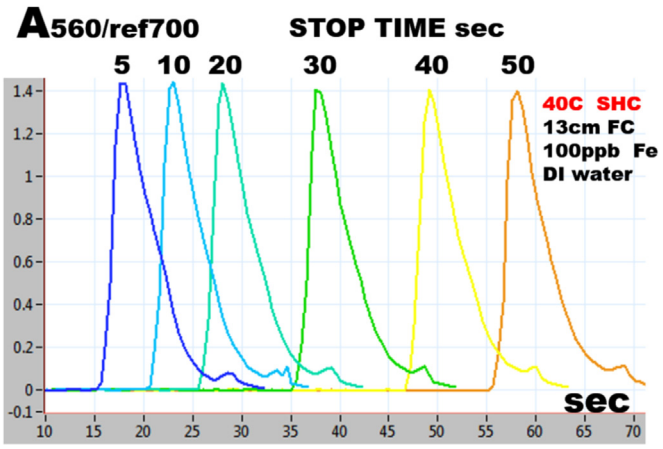

Fig. 4. Effect of stop time in the holding coil on the absorbance of samples of Fe (II), using the SHC method and a S/R mixing ratio of 5/1.

analyte. When optimizing such a protocol, it is practical to investigate and to adjust the key parameters at a higher analyte concentration range in order to avoid confusion that may arise from stray contaminants and/or background noise. As in classical Flow Injection (cFI) the deciding factors in programmable Flow Injection (pFI) using spectrophotometry are;

- Length of the light path of the flow cell
- Incubation time (WAIT time) and temperature
- Injected sample volume (Sv)
- Mixing ratio of sample with reagent (S/R)
- Data collection

The *configuration of the flow cell* (light path 13 cm, internal volume 65 μL) was chosen first and was maintained throughout the optimization procedure. Next, the *incubation time* was changed, by changing the WAIT (INCUBATE) period in the software protocol (Fig. 3) stepwise from 0 to 50 s, while using a Sv of 300 μL and S/R mixing ratio of 5/1. The recorded peaks shown superimposed in Fig. 4 reveal that the incubation is complete after a stop time of 5 s. This WAIT time was then used in all subsequent experiments.

The temperature of the reacting mixture was kept at 40 °C by thermostating the holding coil, only as a precaution, because the rate of Fe chelate with ferrozine is very fast even at room temperature [5–10].

An increase in the *injected sample volume* (Sv) increases the sensitivity of all FI measurements, in a linear fashion, until the $S_{1/2}$ value is reached, above which the increase in peak height continues in an asymptotic fashion until about 5 $S_{1/2}$ volumes [5,15]. By changing the injected sample volume from 50 to 350 μL, evaluation of the superimposed peaks (Fig. 5) revealed an $S_{1/2}$ value of 120 μL. Since not much is gained in terms of sensitivity beyond Sv 300 μL, this latter volume was used in all subsequent experiments.

The mixing ratio of the sample with the reagent (S/R) is controlled by the difference in the volume dispensed by P1 and the volume aspirated by P2 while the central port is connected to the reagent port. The ratio of the flow rates has to be adjusted to the same ratio as the volumes dispensed and aspirated by the pumps. By applying the flow volumes and flowrates expressed in microliters (μL) and depicted in Fig. 6A, a mixing ratio of S/R = 5/1 is achieved. This ratio yields the top peak in Fig. 6B. Decreasing the sample reagent mixing ratio, results in peak broadening and a reduction of the sensitivity of the assay (Fig. 6B).

The optimized conditions for the pFI SHC method, summarized in the assay protocol (Fig. 3) were used to perform two sets of calibration experiments using Fe (II) standards in the range 0–100 ppb Fe, and S/R = 5/1 mixing ratio. One set of standards was prepared in 0.01 M HCl using DI water, while the other set simulated sea water samples by

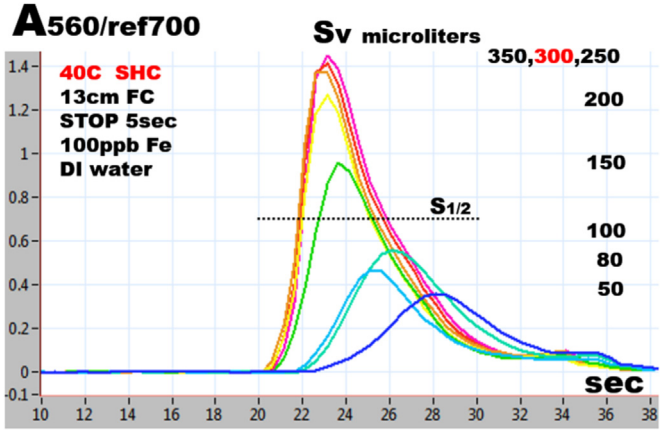

Fig. 5. Effect of changing the sample volume (Sv) on peak shape and height of Fe (II) using the SHC method and a S/R mixing ratio 5/1.

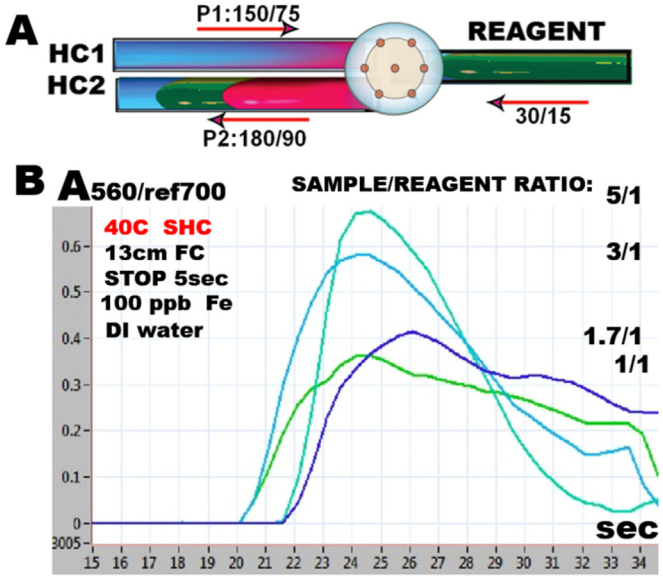

Fig. 6. (A) Schematic of the SHC method. A sample is being dispensed into HC2 while a reagent aspirated from the reagent reservoir by different in the pumping rates. (B) Effect of the S/R mixing ratio on peak height using the SHC method.

using 0.01 M HCl in a 3.5% (w/w) NaCl matrix. The influence of the NaCl matrix is twofold: it produces a dip in the baseline caused by the refractive index gradient between the sample and reagents and it also results in a delay in position of peak maximum (Fig. 7).

In summary, the optimized assay protocol (Fig. 3) was used to prepare calibration curves using standards prepared in deionized water, 3.5% NaCl and sea water. The results are summarized in Section 3 and in Table 1.

2.5. Development and optimization of the SFC method

The key parameters, optimized for the SHC method i.e. sample volume (Sv) and mixing ratio (S/R) are also suitable for SFC assay protocol, and were therefore included in the assay script (Fig. 8). For the SFC method, however, an additional parameter has to be optimized: the positioning of the sample zone within the flow cell [17]. In order to maximize the sensitivity of the assay, the reaction mixture has to be trapped within the flow cell in such a way that the centroid of the sample zone is in the center of the beam of light. This position is defined by the return volume (Rv), which is computed from the time that elapsed from the sample entering the flow cell and the time at which the peak maximum has been recorded in the SHC mode (Fig. 7). At a

Table 1
Summary of methods performance.

Method	SHC	SFC	Manual
Sensitivity			
mAU/ppb Fe	6.2	5.9	4.8
mAU/nM Fe	0.34	0.33	0.27
Limit of detection			
ppb Fe	3.10	0.57	0.57
nM Fe	55.3	10.2	10.3
Reproducibility %	1.9	0.8	3.8
Sample/Reagent	5/1	5/1	4/2
Molar Absorbance			
AU × 10^4	3.10	3.05	3.12
Reagent/Assay mL	0.03	0.03	2.0
Samples/h		90	40

Notes: 1/Data are for assays conducted on samples acidified (0.01 M HCl) 3.5% NaCl. 2/ Molar absorbance is computed for 1 M Fe-ferrozine complex solution in 1 cm light path flow cell corrected for S/R dilution and measured at 560 nm. 3/Linear working range was tested up to 100 ppb Fe or 2 µM Fe. 4/LOD is based on 3 × 6.4 mAU for SHC, 3 × 1.1 mAU for SFC, 3 × 0.64 mAU for Manual measured when using 13 cm light path flow cell. 5/Reproducibility is based on the typical data obtained at 5 ppb or 90 nM.

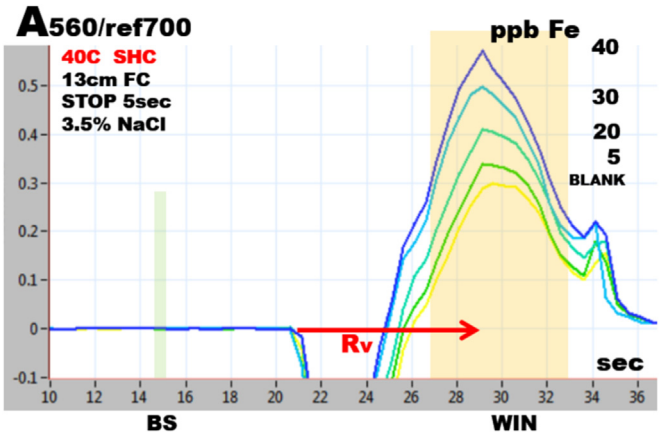

Fig. 7. Response curves obtained with the SHC method using standards prepared in acidified (0.01 M HCl) 3.5% NaCl and the windows used to find the peak maximum (WIN) and baseline (BS).

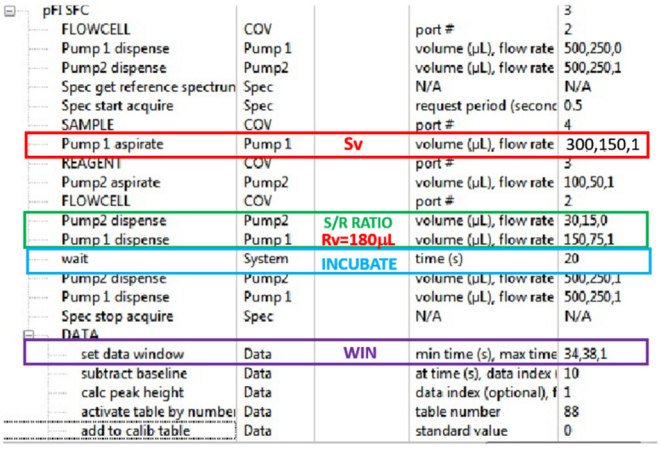

Fig. 8. FloZF program steps for the stopped in flow cell (SFC) method. For details see text.

flow rate of 25 µL/s, and elapsed time of 7.2 s Rv = 180 µL. Finally, the data collection window (WIN, 34–38 s), had to be adjusted in order to accommodate the 20 s long incubation period (Fig. 8).

A series of standard solutions, prepared in acidified (0.01 M HCl) 3.5% NaCl was analyzed by using the SFC protocol (Fig. 8), and the resulting response curves were superimposed in Fig. 9. Because the rate

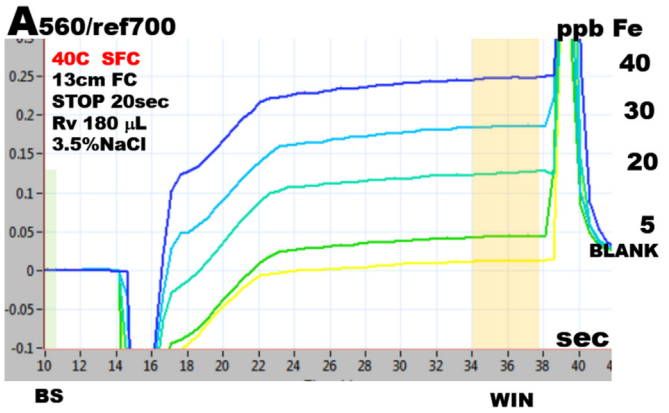

Fig. 9. Response curves obtained with the SFC method using standards prepared in acidified 3.5% NaCl and the windows used to find the peak maximum (WIN) and baseline (BS).

of formation of the ferrozine-Fe (II) complex is very fast, most of the color was formed on the way to the flow cell, at an elapsed time of 26 s. In order to ensure that a stabilized response was measured the data collection window (WIN) was placed between 34 and 38 s and baseline value (BS) was collected at after a 10 s elapsed time. The difference between these absorbance values was used to construct the calibration graphs (shown as "$A_{560/ref700}$") and to analyze the Fe concentration in the samples. The calibration graphs obtained in 0.01 M HCl, in acidified (0.01 M HCl) 3.5% NaCl, and in acidified (0.01 M HCl) sea water are discussed in Section 3, where the SFC method is compared to the manual method and used for determination of total Fe in a sea water matrix.

3. Results and discussion

3.1. Calibration of the SHC, SFC and manual methods

A calibration graph and the information associated with it is the ultimate proof of validity of any analytical method. Therefore, the three methods were validated by using the same set of iron standards prepared in the following three matrix solutions:

- acidified (0.01 M HCl) deionized water
- acidified (0.01 M HCl) 3.5% (w/w) NaCl
- acidified (0.01 M HCl) sea water

These solutions were analyzed using the optimized SHC and SFC protocols as well as the manual method.

The results are summarized in Figs. 10–12 and in Table 1.

These results show that the calibration curves of the SFC (Fig. 11) and the manual method (Fig. 12) are for all practical purposes the same, when corrected for the different S/R dilution factors (Table 1).

While there is a very small offset, between the matrices, the slopes are unaffected by the salinity of the samples (Fig. 11), which makes them ideally suited for oceanography as the salinity of sea water particularly in brackish coastal waters may vary considerably.

In contrast, the SHC method exhibits larger offsets and slightly different slopes for different matrices (Fig. 10). These effects are probably due to the influence of the refractive index boundary between the DI carrier and the seawater + reagent mixture as it moves through the flow cell, at the same time as the peak maximum is being recorded.

Remarkably, all the calibration curves for standards in 3.5% NaCl in Figs. 10–12 and, exhibit slopes that, when corrected for S/R dilution, the length of flow cell light path and the concentration of iron, yield 1 M ferrozine Fe AU values that are very close to the reported molar extinction coefficient (Table 1). The implications of these findings are discussed in Section 4.

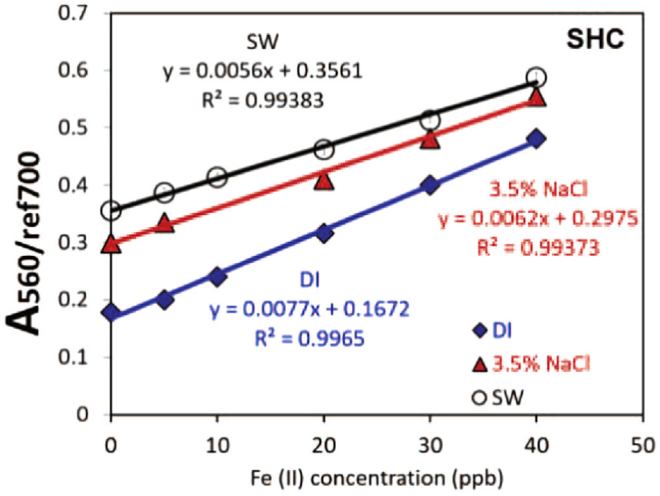

Fig. 10. Calibration graph obtained by the SHC method using standards in 0.01 M HCl-DI water (blue diamonds), standards prepared in 0.01 M HCl-3.5 % NaCl (red triangle, data from Fig. 7), standards prepared in acidified (0.01 M HCl) sea water (black circle). S/R = 5/1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

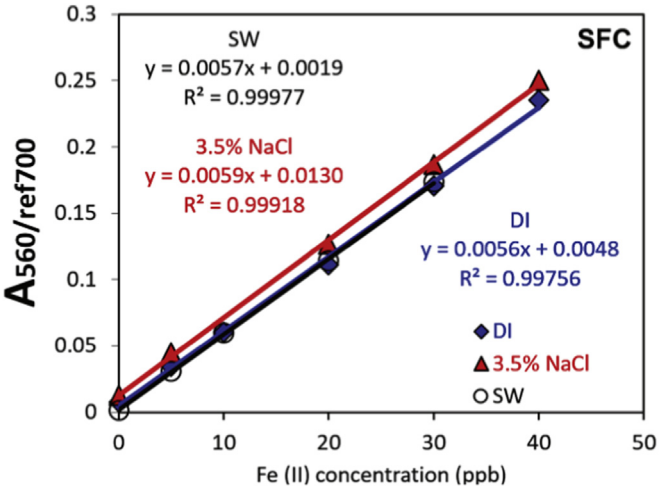

Fig. 11. Calibration graph obtained by the SFC method using standards in 0.01 M HCl- DI water (blue diamonds), standards prepared in 0.01 M HCl-3.5 % NaCl (red triangle, data from Fig. 9), standards prepared in acidified (0.01 M HCl) sea water (black circle). S/R = 5/1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Comparison of the SFC and manual methods for determination of Fe in sea water of different salinities

Fe (II) was determined using both the SFC and manual methods using seawater samples prepared as described in Section 2.2. The results (Table 2) show that the agreement between the two methods is within the analytical uncertainty of each method at each salinity level. It should be noted that in each case the values have been corrected for the methodological blank of the particular method which were independently assessed at 53.0 nM (manual) and 13.8 nM (SFC), respectively. The higher blank of the manual method is ascribed to the Fe blank within the ferrozine reagent and the higher concentration of that reagent used in the manual method.

4. Conclusions

Key components comprising the traditional flow injection analyzers are always arranged in the same linear sequence: injector, confluence

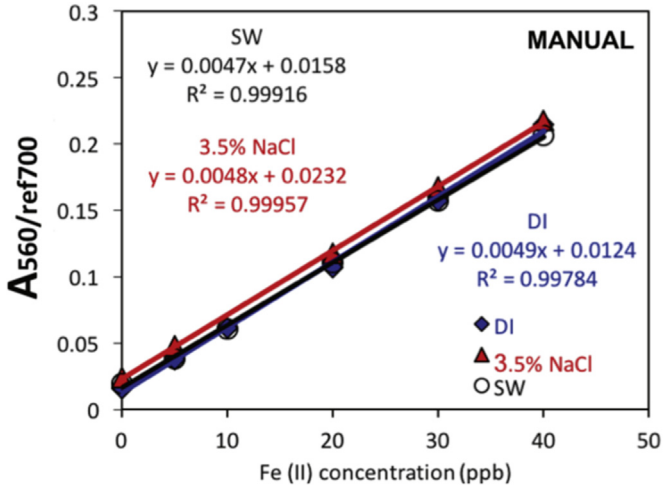

Fig. 12. Calibration graphs obtained by the manual method using standards in 0.01 M HCl-DI water (blue diamonds), standards prepared in 0.01 M HCl-3.5 % NaCl (red triangle), and standards prepared in acidified (0.01 M HCl) sea water (black circle). S/R = 4/2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Blank corrected results from SFC and Manual methods.

Sample	Contents	SFC (nM)	Manual (nM)[a]
Sample #1	80% SW	160.0 ± 2.5	165.6 ± 8.3
Sample #2	50% SW	172.1 ± 2.1	169.8 ± 8.5
Sample #3	20% SW	171.4 ± 2.8	172.9 ± 8.6
Sample #4	0% SW	173.0 ± 0.7	165.4 ± 8.3
Sample #5	1.75% NaCl	195.6 ± 3.3	199.7 ± 9.6

[a] Since the manual method only permits a single estimate, the standard deviation of the results for this method are based on the standard deviation of standards prepared in a similar manner and run through the manual method.

point, mixing coil and flow cell. In order to optimize an assay, these components have to be physically modified. Changing the injected sample volume requires changing the sampling loop, changing the incubation time requires changing the length of the mixing coil or the flowrate, and changing the sample/reagent ratio requires changing the tubing in the peristaltic pump. The cFI manifold configured for single reagent assay also has to be reconfigured in order to accommodate a two reagent assay.

In contrast, the key components of the pFI analyzer, the holding coils, and the flow cell are arranged around the LOV module within which the injector and the confluence point are integrated. Optimization of an assay is performed by means of flow programming, without any need for reconfiguration of the flow system. Single and multiple reagent assays can be accommodated by the same LOV manifold [18].

Optimization of the assay protocol in pFI mode, executed by a software script, is not only convenient but also efficient, as demonstrated in this work. The optimized SFC protocol yielded a calibration line with a slope of 0.0059 AU for 1 ppB Fe in acidified 3.5% NaCl in a 13 cm long flow cell (Fig. 11, Table 1). This value, recalculated for 1 cm flow cell, and 5/1 sample reagent mixing ratio, yields AU = 3.05×10^4 for 1 M solution of Fe-ferrozine complex, which is very close to the molar extinction coefficient of $\varepsilon = 2.86 \times 10^4$ published in literature [7]. Equally close agreement of molar absorbance values were found for SHC and the manual method (Table 1). This finding confirms that (1) the ferrozine Fe (II) complex formation reached equilibrium, (2) the long light path flow cell was entirely filled with the reacted iron (II) ferrozine complex, and (3) the optimization of the assay protocol minimized the dilution of the sample by the reagent and carrier during sample transfer into the flow cell. In other words, the sensitivity of ferrozine-Fe (II) assay was maximized, reaching the theoretical limit, by careful optimization of the assay protocol.

There are three unique, unprecedented features of the programmable Flow Injection SFC technique:

- It uses only 30 μL of reagent per assay.
- The calibration response is independent of the salinity of the samples.
- The instrument does not use peristaltic pumping.

This work is the first application of programmable Flow Injection to a real life assay. We hope that this communication will serve as inspiration for further application of this novel methodology in chemical oceanography and in instrumental analysis, not only because of its novelty, but due to its advantages compared to methods presently in use.

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