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Simultaneous multiple injection in monosegmented flow analysis

Vanessa O. Brito, Ivo M. Raimundo Jr.*

Instituto de Química, UNICAMP, CP 6154, CEP, 13083-970 Campinas, São Paulo, Brazil

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Abstract

A flow approach for simultaneous multiple injection (SMI) in monosegmented flow analysis (MSFA) is described. In this approach, the sample and the reagent (or other solution, such as a diluting fluid) are simultaneously injected into the reaction coil of a monosegmented flow analyser. The monosegment is homogenised while it is carried towards the detector. The sample dilution procedure is not based on a gradient concentration pattern and dilution factors up to 150 were obtained by using hydrodynamic sampling. The system was applied to perform sample dilution in the determination of glucose in blood plasma. The results showed good correlation with those obtained by the Clinical Hospital of UNICAMP. The SMI approach was also applied to add reagent to the sample in nitrite determination in natural water, providing a methodology that has a sampling frequency of 72 h^{-1}, a relative standard deviation of <2%, at 60 $\mu g\ l^{-1}$ $N\text{-}NO_2^-$, a linear response range up to 260 $\mu g\ l^{-1}$ and a 3 s limit of detection of 5 $\mu g\ l^{-1}$ $N\text{-}NO_2^-$. Thus, the sensitivity is close to that of the manual reference method. Recovery tests carried out with sea water samples also showed that MSFA overcomes the Schlieren effect, without needing any special procedure.

Keywords: Monosegmented flow analysis; Simultaneous multiple injection; Schlieren effect

1. Introduction

Monosegmented flow analysis (MSFA) [1,2] is characterised by the injection of the sample into the reaction coil between two air bubbles. This procedure allows long residence times with insignificant sample dispersion and, therefore, high sensitivity can be achieved even though a method based on relatively slow reactions is employed. In addition, the sampling frequency is maintained as high as in flow Injection analasis systems because several samples can be processed simultaneously.

In MSFA systems, the reagents are often mixed into the sample by differential pumping before the filling of the sample loop [3–5], as described in the original paper [1]. This procedure provides an efficient mixture between sample and reagents, but the precision of the sampling is quite dependent on the flow rate of each solution, which can oscillate due to variations in the peristaltic pump velocity and to pumping tube wear. Recently, the addition of reagents after the injection of the sample has been proposed. However, the requirement for bubble detection imparts a certain complexity to the flow analyser [6].

The sliding central bar injector (or proportional injector) developed by Bergamin and co-workers [7] is a simple, low cost and versatile device. It is

*Corresponding author. Tel.: +55-19-788-3136.

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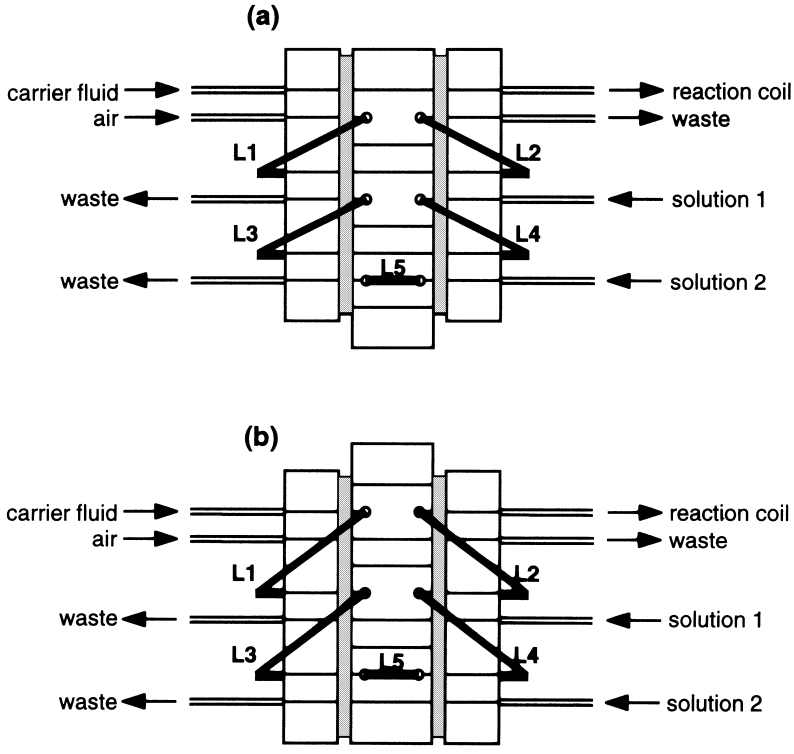

Fig. 1. System for simultaneous multiple injection, showing sampling valve in (a) sampling position and (b) injection position.

generally made of acrylic and is constituted by independent sections, each of them equivalent to a three-way solenoid valve [8], which are used to sample different solutions. This injector has been employed to implement different flow manifolds in FIA, such as merging zones [9], intermittent flow [10], zone trapping [11], and detector relocation [12]. Furthermore, it has been also employed to perform the injection of the sample monosegment in MSFA [1,3–5], by using two sections.

In the monosegmented approach, a third section of the proportional injector can be used to sample another solution, such as a reagent solution or a diluting solution. In this case, the extra loops are assembled like the air loops, as shown in Fig. 1. So, the monosegment is inserted into the reaction coil as a sequence composed of air, solution 1, solution 2, solution 1 and air, where solution 1 can be the reagent (or diluting fluid) and solution 2 can be the sample or vice-versa. The mixing between the solutions takes place while the monosegment is carried towards the detector. More than three sections of the injector, if available, can be used to sample other solutions, which can be simultaneously injected into the reaction coil. In consequence, the procedure described in this paper is called simultaneous multiple injection (SMI). This approach is initially evaluated for efficiency of mixing between the injected solutions by looking at the homogeneity of the monosegment.

Although one of the features of the MSFA systems is the low sample dispersion, which improves the sensitivity, sometimes it is necessary to dilute the sample in order to take its concentration within the useful linear range of specific methodology. This is the case often found in clinical chemistry, where the concentrations of the analytes in blood are frequently high. In FIA systems, the dilution of the sample is carried out by on its dispersion in the carrier fluid. The simplest way to cause sample dilution consists in the injection of a small volume or in increasing the diameter and/or the length of the reaction coil. However, these procedures sometimes are not enough to

adjust the dilution of highly concentrated samples and, in addition, decreases sample throughput as a consequence of the high dispersion imposed on the sample. Many attempts have been made to overcome this problem, such as the use of a confluence point [13], zone sampling [14,15], flow splitting [16], differential pumping [17], cascade dilution [18], pre-valve dilution [19], dialysis [20] and electronic dilution [21]. All of these dilution procedures are based on concentration gradients, generated by the injection of the sample into a carrier fluid. So far, however, concentration gradients are still incompatible with MSFA systems, since the sample (plus reagents) stays between the two air bubbles which maintain the homogeneity of the solution and restrict its dispersion. Besides, simple flow diagrams, such as those that employ a confluence point [22–24], are not also suitable for MSFA because they break the monosegment. Thus, a procedure based on both SMI and hydrodynamic sampling [25] is described in this work to perform sample dilution for determination of glucose in blood plasma.

A priori, manual methods have higher sensitivity than flow injection methods, since the sample dilution can be maintained as little as possible. In other words, in manual methods a small volume of a concentrated reagent is often added to a large volume of sample, allowing high sensitivity. As pointed out previously, MSFA is suitable to determine species that occur in low concentrations, since the sample dispersion is very low even for high residence times. Furthermore, by employing SMI, sample and reagents (for example, solutions 1 and 2 in Fig. 1, respectively) can be injected in a volume ratio equal to those used in the manual method. Therefore, considering that a monosegment simulates a small volumetric flask, a sensitivity close to the manual method can be expected if this approach is employed. This statement is demonstrated in this work for the determination of nitrite in natural waters.

The Schlieren effect arises in FIA systems due to concentration gradients that occur when a sample of high ionic concentration is injected in a carrier fluid of low ionic strength (or vice versa). It is characterised by a negative peak that appears before the analytical signal, causing a lowering of the signal and, therefore, erroneous results. Many attempts have been made in FIA to overcome this serious problem, which include dual wavelength measurements [26,27], adjustment of the ionic strength of the carrier fluid [28], injection of large sample volumes [29] and salinity compensation manifolds [30]. These procedures have disadvantages such as the need to use a diode-array spectrophotometer [27], the necessity of an algorithm for peak height correction [26], use of large volume of sample [29,30] and dependence on the sample ionic strength [28,30]. In MSFA, as indicated above, there is no concentration gradient in the sample monosegment. So, the Schlieren effect is expected to be minimised or even eliminated in this system, without the necessity of a special experimental procedure or data treatment. The determination of nitrite in sea water was employed in this work to demonstrate the feasibility of the MSFA to overcome the Shlieren effect.

2. Experimental

2.1. Reagents and solutions

Analytical grade reagents and deionised water were always used to prepare the solutions. A 1.0×10^{-2} mol l^{-1} potassium permanganate solution was prepared daily.

Cr(VI) reference solutions from 50 to 250 mg l^{-1} were prepared by dilution of an 1000 mg l^{-1} Cr(VI) standard stock solution ($K_2Cr_2O_7$). A 0.25% (w/v) diphenylcarbazide (DPC) solution was prepared in 25% acetic acid and a 2.0 mol l^{-1} sulphuric acid solution was prepared by dilution of the concentrated acid.

β-D-glucose reference solutions from 20.0 to 400 mg dl^{-1} were prepared in deionised water. A 0.01 mol l^{-1} PIPES (1,4-piperazinediethanesulfonic acid) carrier buffer solution (pH 7.2) was prepared by dilution of the Merck solution (catalogue No. 14144). Merck reactive solution (catalogue No. 14143) used for glucose determination by the GOD-PAP method, was diluted 40 times with Merck solution No. 14144 before use.

Nitrite reference solutions from 20 to 300 μg N-NO_2^- l^{-1} were prepared from a 250 mg N-NO_2^- l^{-1} stock solution ($NaNO_2$), previously standardised as recommended [31]. A reagent solution containing 8.5% phosphoric acid, 1.0% sulphanilamide and 0.1% *N*-1-naphthylethylene diamine were prepared as described elsewhere [31].

2.2. Procedure

2.2.1. Evaluation of the sample monosegment homogeneity

An automated monosegmented flow analyser described elsewhere [6] was employed, with a PTFE reaction coil of 1.6 mm internal diameter, 1.5 m long. Deionised water was used as carrier fluid at a flow rate of 2.0 ml min^{-1}. Fig. 1 shows the sampling valve arrangement used to perform the SMI. Air bubbles defining the monosegment (loops L1 and L2) had a volume of 90 µl. Deionised water was also used as diluter, filling loops L3 (330 µl) and L4 (50 µl). A potassium permanganate solution (20 µl) was injected through loop L5. Absorbance measurements were made at 525 nm by employing a home-made diode array spectrophotometer [32].

2.2.2. Evaluation of the SMI with hydrodynamic sampling

The same analyser described above was employed. However, 2.5 µl of Cr(VI) reference solutions were injected at a frequency of 90 h^{-1}, by employing hydrodynamic sampling, assembled as shown in Fig. 2. An acrylic block, in which holes of 0.5 mm of diameter was made according to Fig. 2, was used as the sampling loop. Sulphuric acid and DPC solutions were added to the sample monosegment through an automatic reagent addition module [6], at flow rates of 0.05 and 0.07 ml min^{-1}, respectively. Absorbance was measured at 540 nm.

2.2.3. Determination of glucose in blood plasma

A glass reaction coil with an internal diameter of 1.6 mm was employed. Hydrodynamic sampling was employed to mix 5.0 µl of sample to 220 µl of diluting solution (carrier fluid). The reagent solution was added at a flow rate of 0.16 ml min^{-1} through the automatic reagent addition module [6]. The carrier flow rate was maintained at 2.0 ml min^{-1}, allowing a sample residence time of 6.5 min and a sample frequency of 90 h^{-1} [6]. Absorbance measurements were made at 620 nm.

2.2.4. Determination of nitrite in natural waters

A manually operated manifold was employed to perform this determination. Sampling was carried out according to Fig. 1, employing air loops (L1 and L2) of 30 µl, sample loops L3 and L4 with a total volume of 500 µl and reagent loop L5 of 20 µl. A polyethylene reaction coil with an internal diameter of 2.0 mm and length of 3.0 m was employed. Deionised water was used as carrier fluid at a flow rate of 3.3 ml min^{-1}. Absorbance was measured at 543 nm with a spectro-

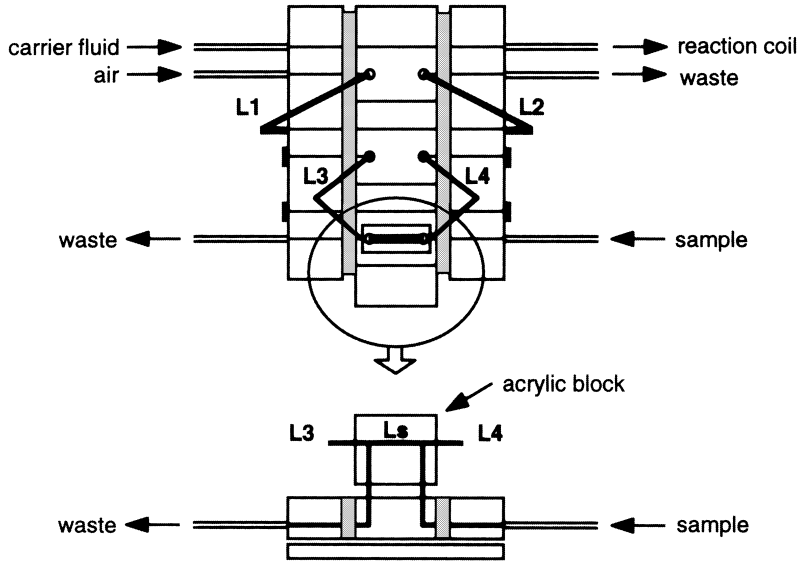

Fig. 2. System for dilution of the sample employing SMI and hydrodynamic sampling (the diluting solution must be the carrier fluid).

photometer (Micronal, model B832) equipped with a 10 mm optical path flow cell. Analytical signals were recorded with a potentiometric recorder (ECB, model RB101).

3. Results and discussion

Monosegmented flow analysis was implemented by mixing previously the sample and reagents because it was realised that the mixing process would be efficient only if a mixing chamber could be employed [1]. However, the air bubbles cause turbulence in the sample monosegment, similar to the effect seen in segmented continuous flow analysis. Thus, when simultaneous multiple injection is employed, the mixing between the solutions is achieved as a result of the 'bolus flow' model [33], providing solution homogenisation. The homogeneity of the sample monosegment was experimentally evaluated by measuring its absorbance at different points, after dilution of a potassium permanganate solution carried out using the SMI approach. Table 1 shows the absorbance values obtained in this experiment. The first point means that absorbance was measured as soon as the front of the sample monosegment had filled the flow cell and at the last point (6th) the absorbance was measured just before the second air bubble of the sample monosegment reached the flow cell. The other points for absorbance measurements were equally spaced between the first and the last points. These results indicate good mixing efficiency and sample monogeneity, since they do not differ from the value obtained by injecting the manually diluted solution.

Table 1
Absorbance values obtained for a $KMnO_4$ solution at different points in a 400 μl monosegment

Location	$(A \pm s)$[a]
1	0.388±0.007
2	0.379±0.003
3	0.398±0.003
4	0.402±0.005
5	0.400±0.006
6	0.399±0.006
Manual[b]	0.400±0.005

[a] Average of 10 replicates ± standard deviation.
[b] Sample manually diluted and measurements carried out in the centre of the monosegment.

The volume of sample employed in this experiment (20 μl) is the smallest that can be injected with the sampling valve due to its internal dead volume. So, high dilution factors can be obtained by increasing the volume of the diluting fluid and/or adding this fluid through the reagent addition module [6]. However, both of these procedures have the disadvantage of increasing the volume of the sample monosegment. Therefore, hydrodynamic sampling, as shown in Fig. 2, was employed to achieve high dilution factors without increasing the monosegment volume. Volumes as small as 2.5 μl can be sampled, because the sample volume is determined by the length of the orifice in the acrylic block, eliminating the dead volume of the proportional injector body. In this approach, the carrier fluid must be the diluting fluid, since the two channels of the sampling valve must be closed as shown. It must be noted that the hydrodynamic sampling proposed in this work can also be used in FIA [34], by removing air loops L1 and L2, with the advantage that it uses only one peristaltic pump, instead of two, as described in the literature [25].

Hydrodynamic sampling was first evaluated by employing the reaction between Cr(VI) with DPC, in an acidic medium. The injection of 2.5 μl of Cr(VI) reference solutions containing from 50 to 250 m g l^{-1} Cr(VI) in a monosegment of 380 μl provided the analytical curve that fits the equation $A = -0.01152 + 0.00185C$ (r=0.9990), n=5 where A is the peak height absorbance and C is the concentration of the Cr(VI) reference solution in mg l^{-1}. The injection of 10 replicates of an 100 mg l^{-1} Cr(VI) reference solution resulted in a mean response of to 101.5 mg l^{-1} (sd=2.3 mg l^{-1}), indicating that there is a carry over between samples. This was confirmed by injecting a blank solution after a 250 mg l^{-1} Cr(VI) reference solution, in which a 'concentration' of 7.6 mg l^{-1} Cr(VI) was obtained. Subsequent blank injections provide a Cr(VI) concentration equal to zero. This carry over probably arises from the hydrodynamic sampling, because there are two interfaces of contact between the sample and the carrier fluid, as can be seen in Fig. 2. However, it can be minimised by sequentially injecting 3 or more replicates of the same sample [6]. Alternatively, a washing cycle can be

Table 2
Glucose concentrations (in mg dl^{-1}) in blood plasma obtained by the MSFA system and the Clinical Hospital (CH) of UNICAMP

MSFA[a]	CH[b]
93.6±0.2	102
124.5±1.5	136
108.8±1.1	116
144.7±2.6	153
223.2±1.4	246
132.7±0.3	144
96.9±1.1	106
197.2±1.1	214
72.6±1.4	82
89.3±1.7	96

[a] Average of three replicates ± mean deviation.
[b] Results supplied by Clinical Hospital of UNICAMP.

employed before injecting the sample for the first time.

SMI was also employed in the determination of glucose in blood plasma by MSFA, providing a sample dilution of 45 times. Table 2 shows the results obtained in this experiment and those obtained by the Clinical Hospital of UNICAMP. These data can be correlated according to the equation $\text{MSFA} = (1.983 \pm 1.895) + (0.908 \pm 0.013)\text{CH}$ (r=0.9990), which agrees with previous results [6] and shows the feasibility of the proposed system for sample dilution.

The experimental conditions recommended for manual methods, such as the concentrations of reagents, volume ratio of sample and reagents and reaction time, can be directly applied in a MSFA system which employs SMI. In the determination of nitrite in natural waters, a reagent concentration and a volume ratio of sample and reagent similar to those employed in the manual method [31] were used. However, a reaction time of 3 min, instead of 10 min [31], was employed because this was long enough to reach the steady state signal.

Table 3 shows the slope of the analytical graphs obtained with the injection of different volumes of sample, maintaining the proportion of reagent constant. As can be seen, the sensitivity is almost independent of the sample volume, as was also pointed out in a previous work [6]. This characteristic arises from the fact that the sample has a very low dispersion in a MSFA system. The polyethylene reactor makes a small contribution to the dispersion. However, the lower sensitivity obtained with small sample volumes is mainly due to flow cell cleaning, since this had a volume of 80 μl and was made of glass, which is readily wetted by aqueous solutions. Thus, up to 400 μl of sample is not enough to efficiently clean the flow cell. These facts determine the sampling frequency in the MSFA system. The carry over was evaluated by injecting a blank solution (water) after a 100 μg l^{-1} N- nitrite reference solution. This experiment indicated that carry over is minimised if samples are sequentially injected with time intervals between injections of at least 50 s. Under the conditions employed, the methodology showed the following figures of merit; a sampling frequency 72 h^{-1}, a relative standard deviation <2% (injection of 10 replicates of a 60 μg l^{-1} nitrite reference solution), a linear response range up to 260 μg $N-NO_2^-$ l^{-1} (r=0.9994, n=10) and a 3 s limit of detection of 5 μg $N-NO_2^-$ l^{-1}. This limit is close to that of the reference manual method [31] and similar to those provided by various flow [35–39] and sequential [40] injection methodologies.

Table 3
Influence of the injected sample volume on the sensitivity of the proposed method (the reagent volume was proportional to the sample volume)

Sample volume (μl)	Slope[a]
100	0.736±0.012
175	0.867±0.014
250	0.884±0.017
350	0.927±0.017
400	0.957±0.015
500	0.954±0.016
600	0.975±0.020

[a] Slope of the analytical graph obtained with reference solutions from 20 to 100 μg N-NO_2^- l^{-1} ± standard deviation (n=5).

Table 4
Nitrite determinations (N-NO_2^- in μg l^{-1}) in synthetic samples prepared in deionised water and in 0.60 mol l^{-1} NaCl

Sample	Water[a]	NaCl[a]	Difference (%)
1	26.8±0.8	27.7±0.2	+3.4
2	69.3±0.6	66.7±0.3	−3.8

[a] Average of three replicates ± mean deviation.

Table 5
Recovery test of nitrite in samples of natural waters[a]

Sample	Unspiked (μg N-NO_2^- l^{-1})	Spike (μg N-NO_2^- l^{-1})	Recovery (μg N-NO_2^- l^{-1})
Tap water 1	14.8±0.8	30	44.6±0.5
Tap water 2	34.1±2.6	30	78.9±0.2
Pretinho river 1	<5	40	33.4±0.3
Pretinho river 2	11.5±0.0	40	49.1±0.5
Sea water (Angra)	9.3±3.6	40	48.9±0.4
Sea water (Ubatuba)	<5	40	49.6±0.5

[a] Average of three replicates ± mean deviation.

An analytical curve was constructed by using a set of reference solutions prepared in deionised water; the latter was also used as the carrier fluid. The concentrations of nitrite in synthetic samples, prepared in both deionised water and 0.60 mol l^{-1} NaCl, were determined. Table 4 shows the results obtained. These results indicate that the Schlieren effect due to salinity differences between samples and carrier fluid is minimised in MSFA systems, since there are no concentration gradients in the sample monosegment. Table 5 shows the results obtained in recovery tests performed after spiking samples of different sources with standard nitrite solution. The recoveries for tap water 2 (123%) and sea water Ubatuba (124%) were slightly higher than the maximum value (120%) recommended by Standard Methods for the Examination of Water and Wastewater [31]. However, these values can be acceptable since the concentration levels of nitrite in the samples were very low. As can be seen, MSFA can be considered as a powerful tool to analyse samples of different matrices, because it naturally overcomes problems relating to the Schlieren effect without requiring any special procedures.

4. Conclusions

Simultaneous multiple injection is a promising procedure that can be employed in MSFA. It can substitute differential pumping sampling and can be applied in determinations which need either sample dilution or sensitivity, by adequately selecting sample and diluting (reagent) loops. In addition, MSFA is a very simple alternative to eliminate the Schlieren effect; so, it can be applied to analyse samples of different aqueous matrices, regardless of their salinities.

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