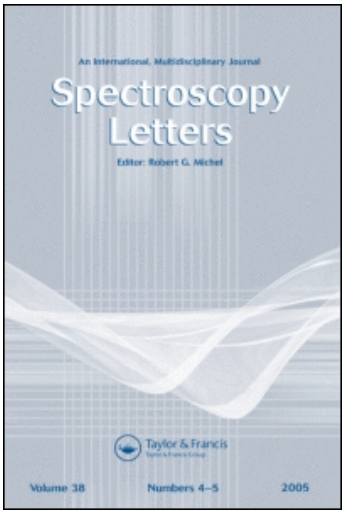

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Multipumping Nitrite Determination in Exhaled Breath Condensate

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Multipumping Nitrite Determination in Exhaled Breath Condensate

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Abstract: A flow system based on multicommutation is proposed for the rapid, clean, and inexpensive determination of nitrites in small volumes of breath condensates. The procedure exploits the colorimetric detection of nitrite with the Griess reagent [0.03% naphthylethylene diamine dihydrochloride (NED), 0.5% sulpfhanilamide, and 3.0% phosphoric acid] in acidic medium at 540 nm correcting the variations of the baseline with measurements at 424 nm. The flow system was designed with a set of solenoid micropumps to minimize sample and reagent consumption and waste generation. The detection limit was estimated as 3.8 ng mL^{-1} (99.7% confidence level) with a linear response ranging up to 500 ng mL^{-1}. The coefficient of variation was estimated as 0.7% for a solution containing 300 ng mL^{-1} nitrite ($n = 9$). Approximately 144 determinations can be carried out per hour, consuming only 678.4 μg Griess reagent and generating 1.184 mL of effluent per determination, thus providing an environmentally friendly alternative

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and a nonexpensive method. The procedure was successfully applied to determine nitrite in breath condensates.

Keywords: Asthma, exhaled breath condensate (EBC), flow injection analysis, multipumping analysis, solenoid micropumps, spectrophotometry

INTRODUCTION

High levels of nitrite have been shown in exhaled breath condensate collected by the patients themselves[1] and could be a home marker for acute asthma.

Recent medical investigations[2–4] have confirmed the increase of nitrite concentration in the exhaled breath condensate of patients with cystic fibrosis and asthma. For these patients, the concentrations of nitrite ranged from 40 to 370 ng mL^{-1}, as compared with normal people with concentration levels lower than 98 ng mL^{-1}.[2] This increase could be due to the reduction of gaseous NO to its metabolites within the increased secretion barrier present in airways of asthma and cystic fibrosis patients. Other authors have found that nitrite levels in healthy people ranged between 140 and 280,[5] 93 and 154,[6] 126 and 191,[7] or 6 and 85[8] ng mL^{-1}. On the other hand, the nitrite levels for allergic patients varied from 121 to 340 ng mL^{-1},[5] for primary ciliary dyskinesia patients from 149 to 177 ng mL^{-1},[6] for cystic fibrosis patients from 20 to 293 ng mL^{-1},[8] and for asthmatic patients from 29 to 254 ng mL^{-1}.[8]

So, nitrite should be conveniently measured in breath condensate in the first stages of the illness to prevent and warn about airway inflammation in patients with respiratory diseases. The Griess reaction has been employed for nitrite determination in exhaled breath condensate, in which after 10 min of color development at room temperature, the absorbance was measured at 550 nm.[9–11] Nevertheless, in our knowledge, no online procedure has been employed for nitrite determination in this kind of sample.

The reduced volume of breath condensate (around 1 mL) makes difficult the application of conventional analysis techniques, in terms of sample availability Analyte determination in small-size samples is a challenge for analytical chemists. Mechanized methods in general use a reduced amount of sample and provide also a reduction of reagent consumption and waste generation.[12,13]

In the development of automated methods, several techniques have been implemented in order to reduce the volume of the different samples used. The first strategy was published by Skeggs in 1957 corresponding with the use of segmented flow,[14] followed by the flow injection analysis (FIA) strategy developed by Ruzicka and Hansen in 1975,[15] the monosegmented flow by Pasquini and Oliveira,[16] sequential injection analysis (SIA) technique proposed by Ruzicka and Marshall,[17] and multicommutation developed by

Reis et al.[18] In this sense, the application of multicommutation principles to clinical determinations could be useful to obtain reliable data from a reduced volume of sample in those samples of difficult acquisition, as in the case of breath condensates.

Automated determination of nitrite has been carried out by using different methods. Fernández-Arguelles et al.[19] describe a selective fluorimetric method for the determination of nitrite in water using a merging zones flow injection system based on the quenching effect produced by nitrite on the fluorescence reagent. Flow injection strategies have been employed by Burakham et al.[20] who proposed a nitrite spectrophotometric detection procedure based on the nitrosation reaction between nitrite and phloroglucinol and by Ensafi et al.[21] who described a catalytic spectrophotometric method based on the catalytic effect of nitrite on the redox reaction between pyrogallolsulfonephthalein and potassium bromate in acidic medium. On the other hand, stopped flow techniques were employed in order to minimize the dispersion of the sample bolus and to enhance the color development, achieving a sample throughput of $45\ hr^{-1}$.[22] Recently, an enzymatic flow analysis, based on the Griess–Llosvay reaction, was developed for the evaluation of nitrite and nitrate in waters. The method employed nitrate reductase and an in-line dilution procedure for these analyses.[23]

Multipumping flow system, based on the utilization of solenoid micropumps, is an attractive strategy for minimizing reagent consumption and waste generation, and it has been introduced as a new device for fluid propelling.[24,25]. It has been demonstrated that solenoid micropumps could replace the peristaltic pump and solenoid valves in the determination of anionic surfactants,[12] cationic surfactants,[26] phenols in water,[27] cyclamate in artificial table sweeteners,[14] and phystic acid in plant extracts[28] by using spectrophotometric determinations. On the other hand, one of the greatest advantages of multipumping, in spite of other strategies, is the insertion of small volumes of reagents and samples, as automatic microburettes or sequential injection devices. So, the aforementioned strategies offer an alternative to obtain low-cost, reliable, and portable flow analysis instruments for *in situ* measurements.

In the current paper, we discuss development of a new micropumping multicommutation analysis (MiMA) strategy, which has been compared with the conventional flow injection analysis approach. Solenoid micropumps were employed to strongly minimize sample and reagent consumption and waste generation. The nitrite concentration in the breath condensate was determined by the colorimetric assay at 540 nm based on the Griess reaction. Analytical performance of the spectrometric method was not deteriorated by the use of reduced amounts of samples and reagents, and many characteristics of the MiMA procedures are better than those achieved in FIA experiments.

MATERIALS AND METHODS

Apparatus

The flow system comprised three solenoid micropumps (Bio-chem 090SP; Boonton, NJ, USA) with a nominal volume of 8 μL per pulse, flow lines of 0.8 mm i.d. PTFE tubing, and one confluence connector. A Pentium 133 MHz microcomputer was employed for system controlling by a parallel interface through software written in Microsoft Visual Basic. A lab-made electronic interface, analogous to that previously described,[13,18] was used to generate the electric potential, and current required to switch on the solenoid micropumps was 12 V and ca. 100 mA. Measurements were carried out with a Hewlett-Packard (Waldbronn, Germany) model 8452A diode array spectrophotometer equipped with a 10-mm optical path and 50-μL inner volume flow cell.

Reagents and Solutions

All solutions were prepared with deionized water (18.2 MΩ cm) and analytical grade chemicals. A 1000 ng mL^{-1} nitrite stock solution from Merck (Darmstadt, Germany) was prepared from the sodium salt, and working solutions (15–500 ng mL^{-1}) were prepared from dilution of the stock solution in water. Griess reagent solution was prepared combining 0.03% naphthylethylene diamine dihydrochloride (NED) from Aldrich (Barcelona, Spain), 0.5% sulfanilamide from Acros Organics (Morris Plains, New Jersey, USA), and 3% H_3PO_4 from Scharlau (Barcelona, Spain). Deionized water was used as a carrier.

Samples of breath condensates were obtained from the Hospital Clínico Universitario de Valencia. Breath condensates were collected using a tube during ca. 20 min. The subject breathes quietly via mouth into the Teflon PTFE tube (55-cm length and 1-cm internal diameter), which was passed through a section of pipe packed with ice. This process yielded 0.5–1.0 mL of viscous breath condensate over the period of 20 min.

Flow Diagram and Procedure

The flow system manifold was designed employing three solenoid micropumps, which were assembled to allow the handling of three different solutions: nitrite samples or standards (S), Griess reagent (R), and water (C). When the solenoid coil of the micropump was energized (ON), a sucking action was carried out, thus permitting the solution insertion into the micropump chamber through the input channel. When the applied voltage was turned OFF, the inner diaphragm went back to rest position and the fluid was dispensed through the micropump output channel.

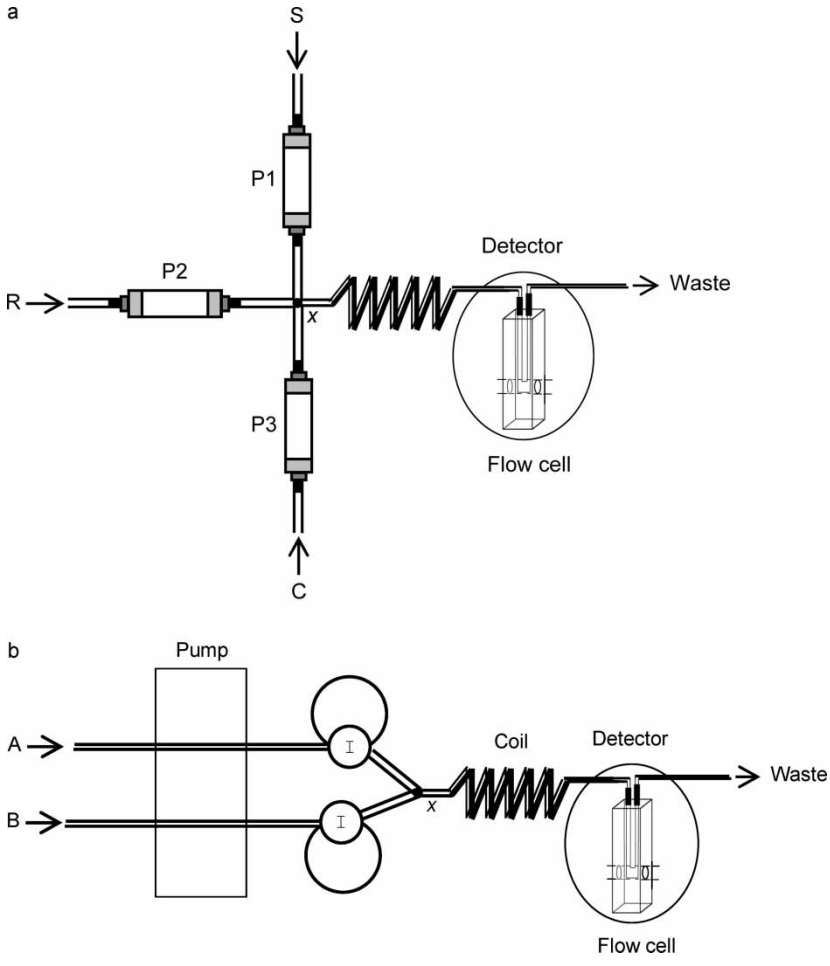

Figure 1. Flow manifolds used for (a) multipumping determination of nitrite. Notes: P_i, solenoid micropumps; spectrophotometric detector (540 nm); coiled reactor (150 cm); x, confluence point; S, sample; R, Griess reagent (0.03% NED, 0.5% sulfanilamide, and 3.0% H_3PO_4); C, deionized carrier water. (b) FIA determination of nitrite. Notes: I, injection valves with 500-μL loop volume each, coiled reactor of 200 cm; x, confluence point; channels A, B, see text.

The solenoid micropumps were arranged as shown in Fig. 1a, employing one device for each solution handled, during 0.1/0.1 s (ON/OFF). The devices were operated at 2 Hz.

The MiMA system was operated as described in Table 1, exploiting the binary sampling approach[13] to mix the solutions. Each step was repeated until completing the number of pulses. Solutions of 256 μL of sample (S) and 128 μL of Griess reagent (R) were sequentially inserted (in the 2:1

Table 1. Solenoid micropumps switching course for nitrite determination[a]

Step	Description	P_1	P_2	P_3	Pulses
1	Mixing of S and R	1/0	0	0	16
		1/0	0	0	
		0	1/0	0	
2	Sample zone removal and reading	0	0	1/0	100

[a]Numbers 1/0 indicate a pulse of the solenoid micropump.

relation) by means of 16 pulses (step 1) in a 150-cm-length coil in which the reaction took place. Sample zone was transported by 100 pulses of carrier toward the detector where transient signal measurements were carried out (step 2). The nitrite levels in the breath condensates were quantified on using external standard curves and measured at an absorbance wavelength of 540 nm with the correction of the baseline at 424 nm. All measurements were carried out in triplicate.

FIA Procedure

A two-channel manifold (Fig. 1b was employed for the FIA spectrophotometric determination of nitrite. A Gilson P2 minipuls peristaltic pump (Villiers-LeBel, France) was used to transport the solutions and two PTFE six-way injection valves (Omnifit, Cambridge, England) were employed to provide appropriate injection volumes of standard or sample solutions and reagent solution.

Channels A and B in the manifold were used to transport standard or sample solution and reagent solution, respectively, water being employed as carrier solution in both cases.

Flexible polyvinyl chloride tubes were employed to provide a flow rate up to 1.8 mL min^{-1} in each channel and were merged by using a Y-tube merging zone in order to provide a good mixing between both solutions. Furthermore, a reaction coil of 200 cm was used to obtain the colored reaction product between the NO_2^- and the reagent and it was passed through the flow cell. Absorbance measurements were done as indicated before.

RESULTS AND DISCUSSION

General Characteristics

The reaction between nitrite and Griess reagent has been exploited in several analytical methods.[29–32] However, the conventional procedures consume a

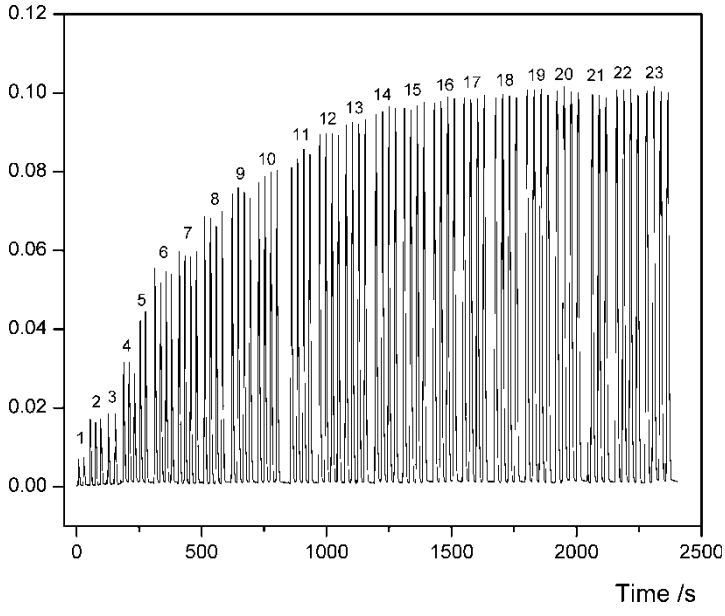

Figure 2. Effect of sample pulses (from 1 to 23) on absorbance signal. Other experimental conditions correspond with 25-cm coil length, 0.1/0.1 s (ON/OFF), 1 : 1 relation between sample:reagent, and 300 ng mL^{-1} nitrite concentration.

considerable quantity of sample. In this study, a rapid and cheap procedure, based on this reaction, has been developed by minimizing sample and reagent amounts by using a flow system with solenoid micropumps.

MiMA approach was selected for solutions handling, by introducing small aliquots of each solution in tandem. Figure 2 shows the effect of the number of sample or standard pulses on the signal response. The effect of increasing the number of sampling cycles was evaluated fixing the sample to the reagent ratio (1 : 1), the concentration of standard solution (300 ng mL^{-1}), and the coil length (25 cm). As can be seen in Fig. 2, the sensitivity of the reaction increases on increasing the number of cycles up to reach a plateau for 16 sampling cycles, indicating that the complete reaction has taken place. Additionally, for a number of sampling cycles higher than 19, widening of peaks was observed and the sampling throughput was diminished. Sixteen pulses were selected as the most suitable volume to complete the reaction achieving the maximum sensitivity and throughput.

The reaction coil length is a critical parameter to achieve a complete reaction and it must be long enough to provide the quantitative reaction between nitrite and reagents. The total formation of colored product is controlled by the coil length, but the increase of the coil length also

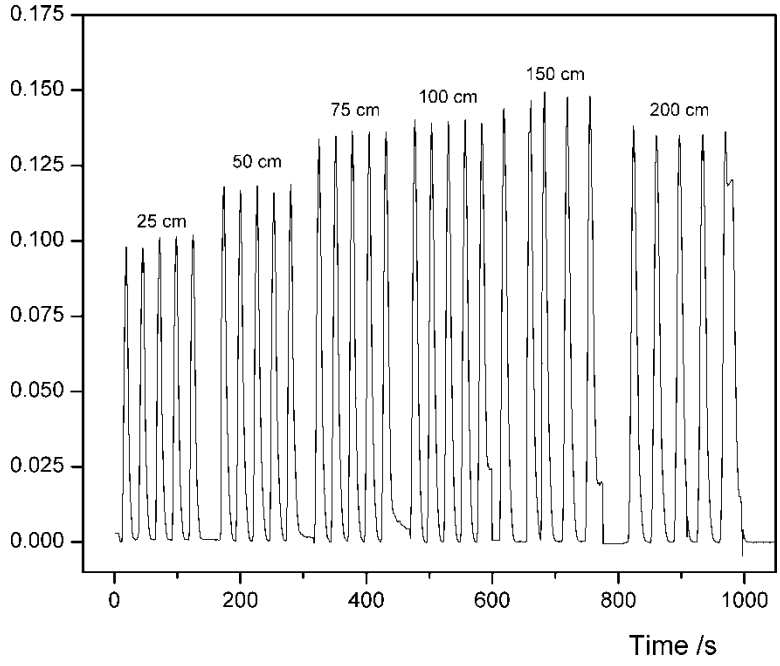

Figure 3. Effect of the coil length on the absorbance of samples. Experimental conditions: 16 pulses sampling, 300 ng mL^{-1} nitrite concentration, 1 : 1 relation between sample:reagent, and 0.1/0.1 s (ON/OFF).

increases the dispersion of the sample plugs, specially when long coils were used. Figure 3 shows the effect of the coil length, from 25 to 200 cm, on the absorbance of 300 ng mL^{-1} nitrite standard solution, for a sample and reagent ration (1 : 1) and 16 sampling cycles. As can be seen, 150-cm coil length supplies the best results in terms of sensitivity because it provides the maximum absorbance signal with reduced sample dispersion.

The effect of different relationships between sample and reagent pulses was evaluated selecting a nitrite concentration of 300 ng mL^{-1}, 150-cm coil length and 16 pulses sampling for attaining a reference signal close to 0.2. Multicommutation made feasible the optimization of sample/reagent ratios by varying the number of pulses of each solution (1 to 3 for sample solution; 1 to 3 for reagent solution). The effect of the insertion method of solutions was studied, observing that alternate introduction of sample and reagent provides better sensitivity than simultaneous introduction of both solutions.

The effect of different ratios between sample and reagent pulses was evaluated, and Fig. 4 shows the absorbance signals obtained. The insertion of solutions was carried out sequentially (pulse by pulse) for all conditions

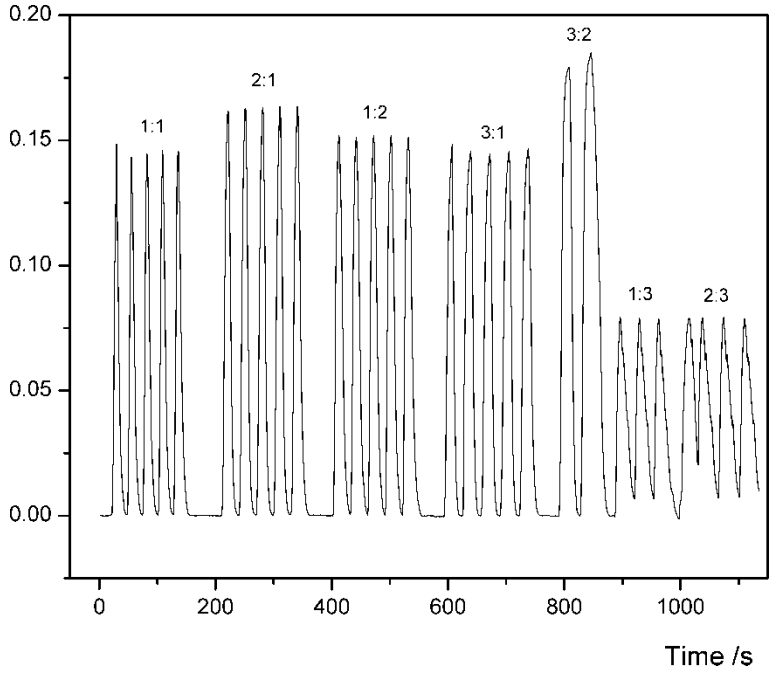

Figure 4. Effect of the relation between sample and Griess reagent (sample:reagent) using alternate insertion. Other conditions: 150-cm coil length, 16 pulses sampling, 300 ng mL^{-1} nitrite concentration, and 0.1/0.1 s (ON/OFF).

studied. The pulse series were researched according to the sequence S.R (see Fig. 1 and Table 1 for details). As can be seen in Fig. 4, the best compromise between sensitivity and productivity was achieved for sample : reagent 2 : 1 proportion volumes.

Analytical Features

System stability and signal repeatability can be seen in Fig. 5, which shows a set of transient signals obtained for several nitrite standards and sample solutions. The limit of detection, established as $3\sigma_b$/slope (σ_b being the standard deviation of 10 blank measurements), at the 99.7% confidence level, was estimated as 3.8 ng mL^{-1} without stopping the flow. Linear response was observed up to 500 ng mL^{-1} and the coefficient of variation was estimated as 0.7% for nine measurements of a solution containing 300 ng mL^{-1} nitrite. About 144 determinations can be carried out per hour, consuming 678.4 μg Griess reagent and generating a waste volume of 1.184 mL per determination.

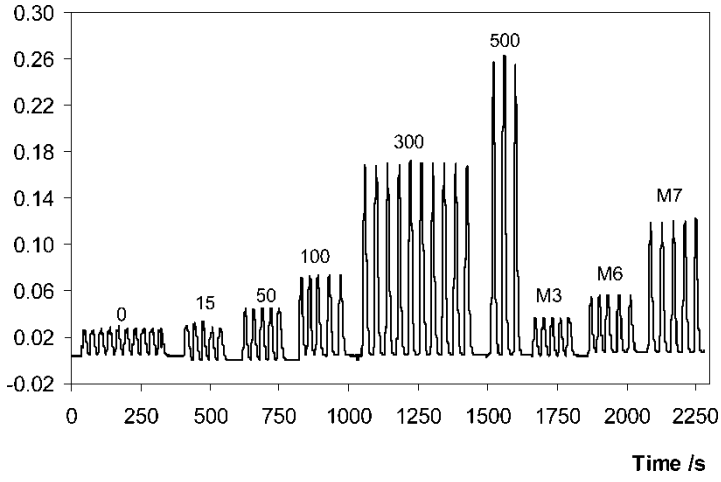

Figure 5. Transient signals obtained for different sodium nitrite standard solutions and samples. Numbers indicate concentrations in ng mL^{-1}. Experimental conditions: 16 pulses sampling, 150-cm coil length, 2 : 1 relation between sample:reagent, and 0.1/0.1 s (ON/OFF).

The correction of the baseline by using dual wavelength measurements was necessary due to the Schlieren effect and the measurement disturbances that could be caused by little bubbles retained in the inner wall of the flow cell.[33]

A comparison of the analytical features achieved by the proposed method and the FIA procedure for nitrite determination is presented in Table 2. The proposed procedure presents a similar linear response range than the FIA one, 2 times better detection limit, and 4.8 times higher sampling rate as compared with the conventional procedure, which additionally involves the continuous introduction of reagents and samples, thus being unsuitable to analyze samples, as breath condensates, with a small size.

On the other hand, the figures of merit of the developed procedure are comparable with those of the method proposed by Ho et al.[2] and clearly better than those of the procedure of McCafferty et al.[34]

The proposed procedure was applied to the determination of nitrite in several breath condensate samples. Table 3 summarizes the data obtained and, as can be seen, values of the same order in all cases were found by FIA and MiMA. The regression between data obtained by the MiMA procedure and those obtained by FIA measurements provided an equation $y = (0.89 \pm 0.02)x + (3 \pm 3)$, with a regression coefficient $R = 0.998$, thus evidencing the good comparability of both approaches, and it confirms the validity of the MiMA nitrite determination in breath condensates.

Table 2. Comparison of the analytical features achieved by the proposed MiMA and FIA procedure for nitrite determination in breath condensates also indicating the features of other proposed methods for nitrite determination in breath condensates

	Flow injection analysis (FIA)	Micropumping multicommutation analysis (MiMA)	Ho et al.[2]	McCafferty et al.[34]
Reagent volume (μL)[a]	500	128	25	25
Reagent cleaning volume (μL)[a]	100	—	—	—
Sample volume (μL)[a]	500	256	100	100
Sample cleaning volume (μL)[a]	100	—	—	—
Waste volume (μL)[a]	7200	1184	—	—
NED (mg)[a]	0.15	0.038	0.03	—
Sulfanilamide (mg)[a]	3.00	0.64	0.25	—
Linear range (ng mL^{-1})	25–500	15–500	23–466	—
Calibration curve	$y = (0.00044 \pm 0.00001)x - (0.0040 \pm 0.0009)$	$y = (0.000476 \pm x + (0.024 \pm 0.001)$	—	—
R	0.9990	0.9997	0.99	—
Limit of detection (ng mL^{-1})	7.8	3.8	—	500
Coefficient of variation (%)	0.6–1.9	0.7–2.1	—	2
Throughput (determination hr^{-1})	30	144	—	—

NED, naphthylethylene diamine dihydrochloride.

[a]Per determination.

Table 3. Mean values and uncertainties (n = 3) for the mechanized determination of nitrites in breath condensates

	FIA (ng mL^{-1})	MiMA (ng mL^{-1})
M1	41 ± 1	47 ± 4
M2	37 ± 1	40 ± 14
M3	20 ± 1	24 ± 16
M4	<LOD	<LOD
M5	<LOD	<LOD
M6	78 ± 1	63 ± 3
M7	211 ± 6	191 ± 9
M8	203 ± 2	185 ± 4

FIA, flow injection analysis; MiMA, micropumping multicommutation analysis; LOD, limit of detection.

The excessively high standard deviation values of some MiMA results can be explained by the breath condensate viscosity, but this problem could be minimized by a previous dilution with water for samples with a high nitrite concentration.

On the other hand, results obtained in additional MiMA experiments concerning recovery studies on samples spiked with known amounts of nitrite, from 40 to 160 ng mL^{-1}, provide values between 95% and 105%. These recovery results were obtained by dividing result found by the sum of real results plus the added amount and multiplying by 100.

CONCLUSIONS

The proposed procedure is a good method for nitrite determination in small-size samples. MiMA analytical features are comparable with those attained by conventional FIA. It can be noticed that the MiMA approach increases the productivity of the laboratory and reduces the sample and reagent consumption between 2 and 4 times as compared with the FIA approach. Moreover, by using MiMA procedures, the cleaning reagent step was avoided and so small waste volumes were generated.

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