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Short communication

Rapid simultaneous determination of nitrate and nitrite on a centrifugal microfluidic device

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ABSTRACT

A centrifugal microfluidic device was developed for the rapid sequential determination of two critical environmental species, nitrate and nitrite, in water samples. The nitrate is reduced to nitrite and the nitrite is derivatized. The analytes are determined spectrophotometrically through the disc with a 1.4 mm pathlength. The detection limits are 0.05 and 0.16 mg L^{-1} for nitrite and nitrate respectively. The use of powdered reagents, the 100 μL sample required and the design of the device suggest that it would be suitable for field use.

1. Introduction

Microfluidic devices have received considerable attention due to their ability to use small amounts of sample and reagents, provide results rapidly and, in some cases, be disposable. The small format of these devices also suggests their suitability for field work. Of particular interest, centrifugal microfluidic devices, also known as "Lab on a CD" [1], need no pumps or external power connections such as those required for electroosomotic flow. These devices or discs fall well into the concept of the ideal micro Total Analysis System (μTAS). One only needs to inject the sample into the reservoir and mount the disc on the rotational assembly. Detection elements may be external to the disc and are usually spectrometric, allowing a device with no physical connection to the disc other than the rotational assembly. The centrifugal device goes through a pre-determined spin cycle, which can do a wide variety of "unit operations" [2] including mixing, sedimentation, metering and liquid movement. Measurements on the disc can be achieved by a variety of detection techniques; however, absorbance and fluorescence are the most common spectrometric techniques.

Nitrogen compounds are naturally found in watersheds; however, high levels of nitrate and nitrite can lead to a wide range of detrimental health effects [3] and ecosystem degradation [4]. Consequently these species are monitored and regulated by agencies such as the World Health Organization (WHO) and Environment Canada [5,6]. Due to the potential for interconversion of nitrogen species, it is desirable that both species be measured simultaneously and at the point of collection. There are numerous published methods for the determination of nitrates and nitrites, based on spectrophotometry, ion chromatography, electrochemistry and enzyme reduction [3,4,7–9]. However, these methods either generate large amounts of carcinogenic waste or are not well suited to field use. In a previous paper in this journal, we reported on a centrifugal microfluidic system that could be used for either nitrite or hexavalent chromium analysis in water samples [10]. The determination of nitrate was not reported at that time as the chemistry is more complex and involves two steps.

This paper reports the demonstration of a rapid method designed for on-site detection of both nitrate and nitrite levels in water samples using a single centrifugal microfluidic device. The classical Greiss method is used for determination [11–13]. The use of powdered reagents allows for long-term storage of the device and negates the need for the transport of liquid reagents to the field. Capillary valves are used to regulate the flow of liquid through the channels in the device, allowing for multiple step reactions. At lower rotational rates, agitation is used to mix the dry reagents and samples, while higher rates initiate the transfer of solution from the inner to the outer reservoir for further reaction. A multi-wavelength technique is employed for spectrophotomet-

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ric determination of the signal to analyze simulated environmental water samples.

2. Experimental

2.1. Reagents

All standards were prepared with distilled deionized water (DDW, 18 MΩ, Millipore Co., Bedford, MA, USA) by mass in containers cleaned with nitric acid. Nitrite solutions were prepared by dilution from a 100 mg L^{-1} NO_2^--N (nitrite as nitrogen) stock solution (SPEX, Metuchen, NJ, USA) to appropriate concentrations. Nitrate solutions were prepared by dilution from a 1000 mg L^{-1} NO_3^--N (nitrate as nitrogen) stock solution (RICCA Chemical Company, Arlington, TX, USA) to appropriate concentrations. All solutions were stored in a refrigerator at 4 °C. The reagents for the derivatization (NitriVer®3) and the reduction (NitraVer®6) reactions were purchased from Hach Corporation (Products 2107169 and 2107249, Loveland, OH, USA) in sealed "pillow packs". According to manufacturer specifications, the nitrite derivatization reagent consisted of chromatropic acid disodium salt, sodium sulfanilate, potassium pyrosulfate, monobasic potassium phosphate, 1,2-cyclohexanediaminetetraacetic acid trisodium salt [14]. The nitrate reduction reagent consisted of cadmium, sodium sulfanilate, potassium pyrosulfate, monobasic potassium phosphate, magnesium sulfate, 1,2-cyclohexanediaminetetraacetic acid trisodium salt [14,15].

A 0.1 M sodium hydroxide (Fisher Scientific, Fair Lawn, NJ, USA) solution was prepared in DDW for the pre-treatment of the fused silica capillaries (25, 50, 75 and 100 μm inner diameter, Polymicro Technologies, Phoenix, AZ, USA). Ethyl alcohol (Commercial Alcohols, Brampton, ON, Canada) was used for cleaning and the pre-treatment of the capillaries.

2.2. Device fabrication

The centrifugal microfluidic discs were designed and fabricated as previously described [10,16,17] using a combination of xurography and computer numerically controlled (CNC) micro-milling. The resulting five layered device contained twelve single cell systems for nitrite analysis and twelve dual cell systems for nitrate analysis. The disc and its components are illustrated in Figs. 1 and 2. The nitrite system is composed of a single reservoir (10 mm in diameter with a 1.4 mm pathlength or depth) for both the derivatization reaction and detection. This is similar to our previously reported system [10]. The nitrate system requires two reservoirs that are connected by a 5 mm length of fused silica capillary. The first reservoir, closest to the centre of the disc, is the reduction cell (11 mm in diameter) and contains the dry reduction reagent. This is the reservoir into which the water sample is injected. The second reservoir serves as both the derivatization and detection cell (10 mm in diameter) and contains the solid derivatization reagent. All dry reagents were added during disc construction prior to the sealing of the top layer onto the device. Prior to embedding the capillary pieces into the device using commercially available 5 min epoxy, they were pre-treated with NaOH solution, EtOH and air to clean the inner walls.

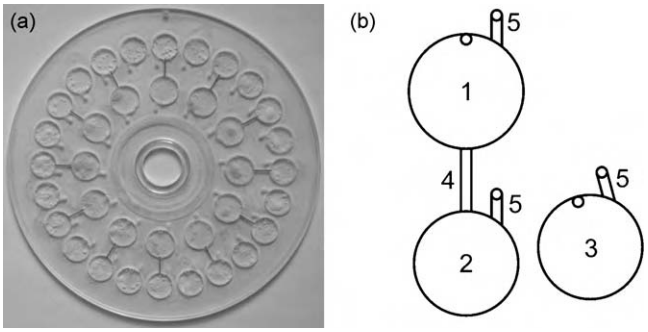

Fig. 1. (a) Fully assembled device for determination of nitrate and nitrite with 12 sets of systems. (b) Schematic of one nitrate system and one nitrite system consisting of (1) reduction reaction reservoir for nitrate containing NitraVer®6, (2) derivatization reaction and detection reservoir for nitrate containing NitriVer®3, (3) derivatization reaction and detection reservoir for nitrite containing NitriVer®3, (4) valve made of a short length of fused silica capillary, and (5) vents.

2.3. Procedure and measurement

The device uses a 100 μL water sample for analysis of each analyte. This volume was added to the first reservoir of each of the nitrate systems (Fig. 1b) and the loading holes were resealed as described by Lafleur and Salin [16]. The device was then mounted onto the rotation system previously described by Duford et al. [18]. Mixing to ensure dissolution was achieved by alternating the direction of rotation every half turn for 180 s at 150 rpm. The device was then spun for 30 s at 700 rpm to transfer the reduced nitrate sample from the inner to the outer reservoir for derivatization. The samples or standards were then added to each of the reservoirs in the nitrite systems, the single reservoir system (Fig. 1b). A second mixing step was executed under the same conditions to derivatize the reduced nitrate samples and the newly injected nitrite samples. The disc was then held at rest for 15 min for the derivatization to take place.

Detection was implemented using the methodology and detection system described previously by LaCroix-Fralish et al. [10].

3. Results and discussion

3.1. Microfluidic flow control

Capillary burst valves were used to control the flow of liquid from the inner reservoir to the second reservoir thereby allowing multi-step reactions. Capillary type burst valves can be fabricated in a variety of ways on microfluidic systems [1]; however, we elected to use embedded capillary tubing due to its relatively good reproducibility and ease of use for prototyping. Prior to finaliz-

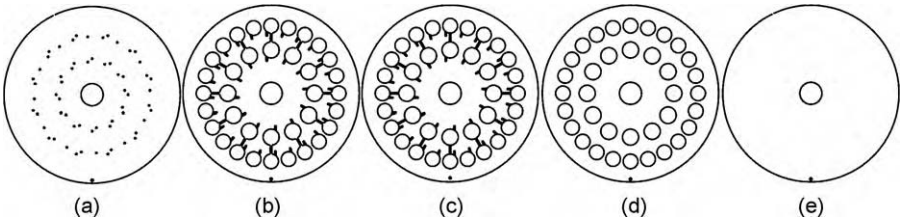

Fig. 2. Layered design of device including, (a) top cover DVD (0.6 mm) with loading holes, (b) double sided adhesive (100 μm) with reservoirs, vents and capillary channel cut out, (c) CD (1.2 mm) making up the main body of device with reservoirs, vent channels and capillary channels milled out, (d) double sided adhesive with reservoirs cut out, and (e) DVD as bottom layer.

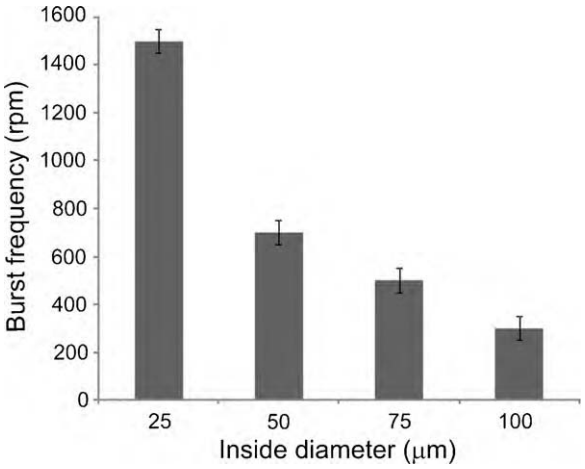

Fig. 3. Burst valve frequency as a function of capillary inner diameter using water. Error bars represent standard deviation, $n = 3$.

ing the design of the device, the burst valve frequencies of the four sizes of capillary tubing were evaluated by the method previously described [17]. The data is provided in Fig. 3 and illustrates a satisfactory narrow range of burst frequencies. The 100 μm inner diameter capillaries were selected for these experiments as they provided the highest flow rate with the lowest and most reliable burst frequency.

3.2. Determination of the required mass of NitriVer®3 and NitraVer®6

One of the advantages to microfluidic devices is the potential to reduce the amount of reagent used. Due to the design of the device, only 100 μL of sample is required for each cell. Full Hach pillow packs supply enough reagents for a 15 mL nitrate sample or 10 mL nitrite sample. Since homogeneity of the dry reagent mixture might be a concern, the weight of reagent required for each reaction was evaluated by measuring the absorbance of a known concentration of analyte with increasing mass of reagent in each cell. The weight of the reducing reagent was varied from 4 to 24 mg for a 3 mg L^{-1} nitrate sample. The highest absorbance was observed at 8 mg, defining the amount of reagent added to each reservoir as 8.0 ± 0.5 mg. The derivatization reagent was varied from 3 to 30 mg for a 3 mg L^{-1} nitrite sample. No significant variation in the absorbance was observed over the range used so 5.0 ± 0.5 mg of reagent was selected for convenience.

3.3. Detection limits

Water samples with only nitrate or only nitrite (i.e. non-mixtures) were used to determine the detection limits of the analytes (Table 1). Using a conventional single wavelength absorbance calculation, the detection limit for nitrate was 0.6 mg L^{-1} (based on the classical three sigma definition). A significant improvement was seen when employing the ratiometric blank estimation technique described by LaCroix-Fralish et al. [10]. Based on the absorbance profile, the absorption wavelength used was the integrated absorbance from 499 to 540 nm and the integrated absorbance from 603 to 624 nm was used for the non-absorbing wavelength. The resulting detection limit was observed to be 0.16 mg L^{-1}. The ratiometric approximation improved the detection limits by minimizing the effect of differences in reflectance, pathlength or lamp drift [10].

Table 1
Summary of calibration parameters for the detection of NO_2^--N and NO_3^--N using the disc-based procedures.

	Single-step reaction NO_2^--N	Multi-step reaction NO_3^-N
R^2	>0.99	>0.99
Linear range (mg L^{-1})	0.05–5	0.16–5
LOD (mg L^{-1})	0.05 ($n = 6$)	0.16 ($n = 11$)
RSD ($n = 5$)	2%	6%

The detection limits achievable by spectrometric standard procedures as described by the WHO [5] are reported to be 0.01–1 mg L^{-1} for nitrate and 0.005–0.01 mg L^{-1} for nitrite. These are significantly lower than the detection limits achievable with this short pathlength microfluidic device; however, these limits are reported for much larger samples measured on bench top instruments. The levels observed for the centrifugal microfluidic device remain lower than the allowable limits and are therefore useful for quantification as well as threshold testing. For nitrate, the WHO imposed a limit 50 mg L^{-1} nitrate [5] which is reported to be equivalent to 10 mg L^{-1} NO_3^--N [6]. Similarly both organizations report limits for nitrite as 3 mg L^{-1} nitrite on a short-term basis or 0.2 mg L^{-1} as nitrite over the long term. These are provisional limits due to the uncertainty of the susceptibility of humans compared to animals. The reported and experimental linear ranges for both nitrate and nitrite are up to 5 mg L^{-1}. If an extension of the linear range is required, dilution can easily be implemented on disc by splitting a larger volume into a diluted and non-diluted series of cells. If lower detection limits are needed, Steigert et al. [19] has demonstrated a convenient method of gaining a longer pathlength in centrifugal systems.

3.4. Simultaneous nitrate and nitrite analysis

Water samples are most often analysed for both nitrate and nitrite so as to determine the total nitrogen content. This centrifugal microfluidic device integrates both the nitrate and nitrite detection systems (Fig. 1) for simultaneous analysis. To quantify the efficiency of the device, a set of solutions containing varying analyte concentration mixtures was prepared as presented in Table 2. The amount of nitrite in the sample is determined directly in the nitrite cell. The determined nitrite concentration is then used to calculate the amount of nitrate by difference, since the nitrate is reduced to nitrite that is indistinguishable from that already present in the sample. The results obtained (Table 2) validate the utility of this device.

Table 2
Results for determination of nitrate and nitrite in simulated water samples.

#	Prepared concentration (mg L^{-1})			Experimental concentration (mg L^{-1})		
	Total N	NO_2^--N	NO_3^--N	Total N[a]	NO_2^--N[a]	NO_3^--N[a]
1	2.00	0.50	1.50	2.01 ± 0.09	0.58 ± 0.06	1.43 ± 0.09
2	2.50	1.00	1.50	2.62 ± 0.09	1.11 ± 0.07	1.51 ± 0.05
3	3.20	1.60	1.60	3.28 ± 0.07	1.67 ± 0.07	1.61 ± 0.03
4	3.00	2.00	1.00	3.10 ± 0.05	2.07 ± 0.10	1.03 ± 0.12

[a] Errors represent one standard deviation from the mean value, three replicates.

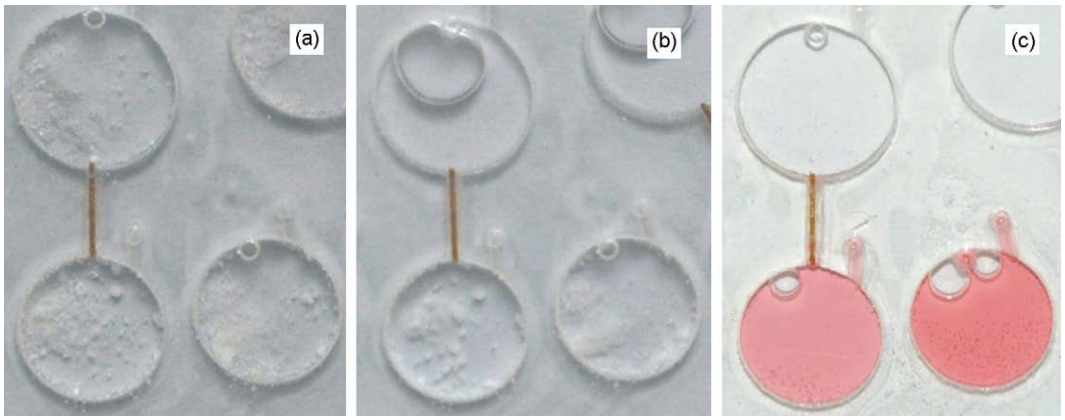

Fig. 4. Photographs of various stages of simultaneous nitrate and nitrite determination where, (a) is the system prior to the addition of sample, (b) shows the nitrate sample after reduction prior to transfer into the second reservoir and prior to addition of the nitrite sample, and (c) system post-analysis.

The operational sequence is illustrated in Fig. 4 with photographs of the dual determination system at various stages. Fig. 4c shows the development of the coloured reagent for the spectrometric measurement of nitrate and nitrite.

4. Conclusion

A simultaneous analysis system for nitrate and nitrite in water sources has been developed and validated using simulated water samples. Only 100 μL of sample is required for each analyte on the centrifugal microfluidic device. Each device has been designed to analyse up to 12 samples of each analyte in approximately 20 min with acceptable detection limits and concentration ranges up to 5 mg L^{-1}. The system appears to be well suited for field use.

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