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Integrated flow analysis platform for the direct detection of nitrate in water using a simplified chromotropic acid method

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This work describes the first use of a direct nitrate analyser using chromotropic acid. A simplified chromotropic acid method eliminating several steps previously associated with this method is employed in the platform. In a sulphuric acid medium, chromotropic acid reacts with nitrate ions and produces a characteristic yellow colour associated with an absorbance band in the visible region ($\lambda_{\text{max}} = 430 \text{ nm}$). The modified method allows for nitrate determination over the linear range 0.9 – 80 mg/L nitrate with a limit of detection of 0.73 $\mu\text{g/L}$ nitrate. Validation was achieved by analysing water samples from various sources including groundwater, trade effluent and drinking water by the modified method and by ion chromatography. The method was implemented on a flow analysis platform incorporating a paired emitter-detector diode (PEDD) as the optical detector. An excellent correlation coefficient of 0.993 was obtained between the modified method and ion chromatography. The modified chromotropic acid method represents a rapid, simple, low cost technique for the direct determination of nitrate in water.

1. Introduction

²⁰ The implementation of the Water Framework Directive in 2000 provides a major incentive for the improvement of water quality in Europe as all European Commission members must achieve “good water status” by 2015, and represents a significant driver towards increased monitoring of the quality of our water systems.¹ The introduction of Horizon 2020 in 2014 will also provide significant actions in environmental monitoring.² There is therefore a growing need for low cost, reliable sensing systems which can be deployed in sufficiently large numbers to ensure that data on key water quality parameters is readily available. This would allow local authorities and other stockholders to make well-informed decisions on the management and protection of our environmental waters.

Autonomous environmental sensors have great potential as a solution to the increasing demand for environmental monitoring.³ By combining microfluidic technologies with colorimetric chemistry, light emitting diode (LED) based optical detection systems and wireless communications, low cost monitoring systems can be developed which are capable of being deployed for extended periods of time. Sensors for nutrients such as nitrate in water and wastewater are particularly important.

Nitrate based fertilisers are applied to promote plant growth, but surplus nitrate entering water bodies is a serious pollutant, causing eutrophication.

Nitrate can also affect health directly, for example through methemoglobinemia, a process by which increased levels of methemoglobin are produced in the blood, resulting in the disruption of haemoglobin and preventing adequate oxygen from reaching the body tissue.⁴ Nitrate levels in various types of water are therefore regulated as per the European Commission’s Groundwater Directive (2006/118/EC) which states a nitrate limit value of 50 mg/L and the Drinking Water Directive (98/83/EC) also stating a nitrate limit of 50 mg/L. Consequently the demand for inexpensive and robust systems for monitoring nitrate has risen sharply in recent years.^{5,6}

Despite the inherent attractiveness of distributed environmental sensing, paying particular attention to nitrate, penetration of autonomous chemical sensing technologies into sensor networks remains disappointingly low in comparison to physical sensing. This is strikingly illustrated by statistics from the Argo project, which was established in 2000 to provide distributed sensor information related to the global marine environment.⁷ Currently, there are 3,558 floats feeding temperature, salinity and pressure (depth) data to the network. In terms of chemical species however, there are only 2 sites monitoring pH, and 27 monitoring nitrate. Of the latter, all are employing the direct optical measurement of nitrate (despite the limited selectivity and rather poor sensitivity of the method) in preference to much more selective and sensitive reagent or separation-based techniques.

Furthermore, monitoring for nutrients such as nitrate in our

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waters is predominantly based on *in situ* manual sampling followed by laboratory analysis using standard laboratory instrumental and/or wet chemical methods. This results in the infrequent monitoring of water at a fewer number of locations than is desirable, as it is time consuming, expensive, non-scalable and requires skilled personnel. Determination of nitrate can prove difficult at times due to the relatively intricate procedures involved, probability of interferences present and the limited detection ranges associated with various methods. Many techniques have been developed and studied for the determination of nitrate including methods involving ultraviolet spectrophotometric screening, nitrate electrodes, cadmium reduction, titanous chloride and hydrazine reduction.⁸ Why are well tried and tested laboratory methods for nitrate making so little headway into practical adoption for autonomous field based instruments? Cost is undoubtedly a major factor, as reagent based analysers are often up to €15,000 per unit (Microlab Ltd. autonomous phosphate analyser retails at ca. €25,000 per unit, including vat, in Ireland; autonomous instruments from YSI, Abb and Hach are similarly priced), mainly due to the need to incorporate expensive fluidic handling components, like pumps and valves, which can comprise approximately 60% of the total component cost.^{9, 10}

Therefore, in order to drive down the cost of ownership of these devices, it is important to keep the fluidic handling requirement as simple as possible, as complex, multistage methods are correspondingly difficult and expensive to implement as well as being less reliable in long-term deployments due to high purchase and maintenance costs. Bearing this in mind, it is not surprising that direct UV-spectroscopy is the current method of choice, despite its analytical performance issues and relatively high cost. This method proves difficult to implement into a microfluidic platform as LEDs in the low UV range (ca. 220 nm) are still in development while LEDs in the range from 240 nm – 300 nm cost approximately US\$200 with relatively limited operating lifetimes. Recently, the Griess method has been investigated in autonomous microfluidic environments with some success, but the method is inherently complex due to the need to incorporate a reduction step (usually via a cadmium reduction column), and estimation of the nitrate concentration through a differential 2-stage measurement.¹¹ Notwithstanding these issues, the Griess method remains popular, and a number of recent articles describe its implementation through various fluidic platforms.¹² Concisely, there is no method available that meets the operational requirements and cost constraints at this present time for the direct determination of nitrate.

The purpose of this study therefore, is to investigate a simple,

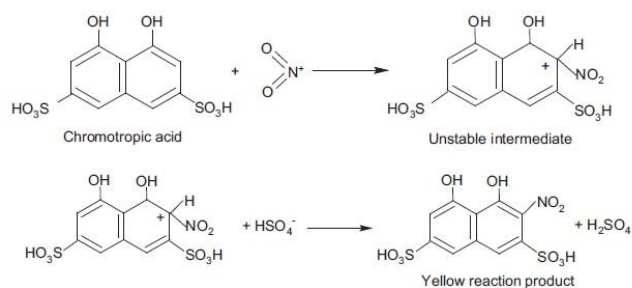


Fig. 1 Mechanism of nitrate detection employing the chromotropic reaction method.

reliable and rapid method for the direct determination of nitrate without the use of columns or expensive materials, allowing the integration of a method into a simple deployable platform for the autonomous monitoring of nitrate. For that reason, a colorimetric technique for nitrate based on the use of chromotropic acid has been examined and optimised.

The method was first described in 1960 by West *et al.* followed by a study performed by Clarke *et al.* for the determination of nitrate in soil.^{13,14} Although a recent study by Bulgariu *et al.* has been carried out describing this method, no single study exists describing the integration of the method into a sensing platform.¹⁵ This paper describes the modification of this method to produce a simple, rapid technique for the determination of nitrate with a wider linear range, lower limit of detection and faster reaction time, allowing for its incorporation into an autonomous monitoring system.

In a sulphuric acid medium, chromotropic acid reacts with nitrate ions to produce a characteristic yellow colour ($\lambda_{\text{max}} = 430$ nm), via the mechanism shown in figure 1. In concentrated sulphuric acid, nitrate ions are converted into nitronium ions which react with the chromotropic acid to form an intermediate which is relatively unstable at high temperatures (ca. 130 °C). This energy is generated by the exothermic reaction produced by addition of the aqueous solution to the sulphuric acid. The proton is then removed and recombines with the hydrogen sulphate ion leading to the reestablishment of the electron delocalisation in the aromatic ring. The exact structure of the product formed as a result of this reaction is still the subject of debate.¹⁵

The first reported methods using chromotropic acid as a technique to determine nitrate included complex procedures such as cooling and heating steps, with added reagents for increased sensitivity, see figure 2.¹⁶ This study however has eliminated this complexity, reducing the method to a one-step process based on a

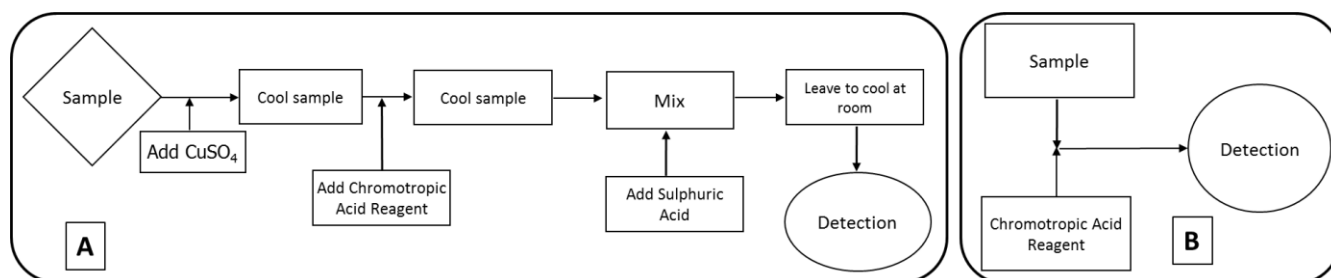


Fig. 2 (A) The conventional chromotropic acid method for direct determination of nitrate, showing the various stages involved in generating the coloured complex; (B) The simplified chromotropic acid method.

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1:1 reagent to sample ratio, which is ideally suited for an automated sensing platform for nitrate.

In this article, a novel automated detection system is introduced incorporating the modified chromotropic method for the direct determination of nitrate to produce a low-cost, wireless optical sensor based on a paired emitter-detector diode (PEDD) as a photometric detector system. Concisely, the PEDD system employs an emitter and detector LED with λ_{max} of 430 nm and 630 nm respectively. The benefits of using the PEDD system as a detection source are its low cost and high sensitivity.¹⁷ The PEDD system has previously been used for a range of chemical detection applications including sensing of pH, phosphate, nitrite and carbon dioxide.¹⁸⁻²¹ This inexpensive, miniature, power efficient detection system achieves exceptional sensitivity while also being remarkably simple to fabricate, making it an ideal detector for coupling with this method.

2. Experimental

2.1 Colorimetric Reagent

The modified method established involves dissolving 0.0294 g of chromotropic acid ($\text{C}_{10}\text{H}_6\text{Na}_2\text{O}_8\text{S}_2 \cdot 2\text{H}_2\text{O}$, Sigma-Aldrich, Ireland) in 100 ml concentrated (96% v/v) sulphuric acid (H_2SO_4 , BDH Laboratory Supplies, UK). When stored under coloured glass and protected from light the reagent is stable for at least one month.

2.2 Deionised Water and Standards

All solutions were made up using analytical grade chemicals. Deionised water from a Millipore Milli-Q water purification system was used throughout the analysis. Stock standards were

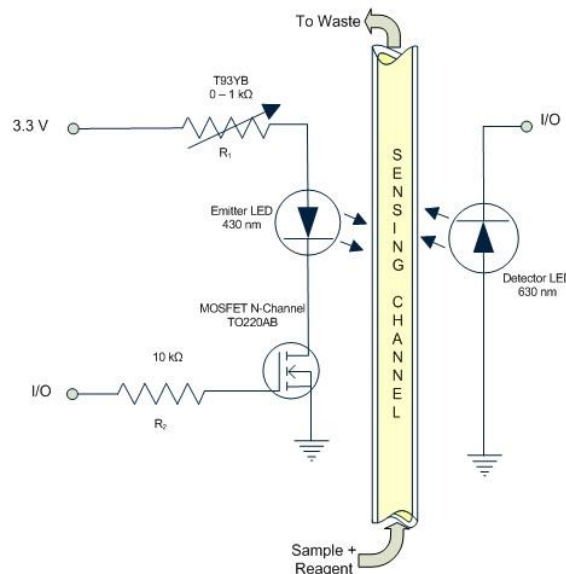


Fig. 3 Schematic of the PEDD detection system

freshly prepared weekly. Nitrate stock standard solution (100 mg/L NO_3^-) was prepared from potassium nitrate (KNO_3 , Sigma-Aldrich, Ireland) that was pre-dried for 1 hour at 110 °C.

2.3 Measurement Procedure

The colorimetric reagent was added in a 1:1 v/v reagent to sample ratio and the solution was allowed to stand for at least 5 minutes in the dark for colour development. The absorbance of the solution was measured at a λ_{max} of 430nm.

Spectrophotometric measurements were performed using a VWR UV-1600PC spectrophotometer. Measurements were recorded using M.Wave Professional software (VWR, Ireland).

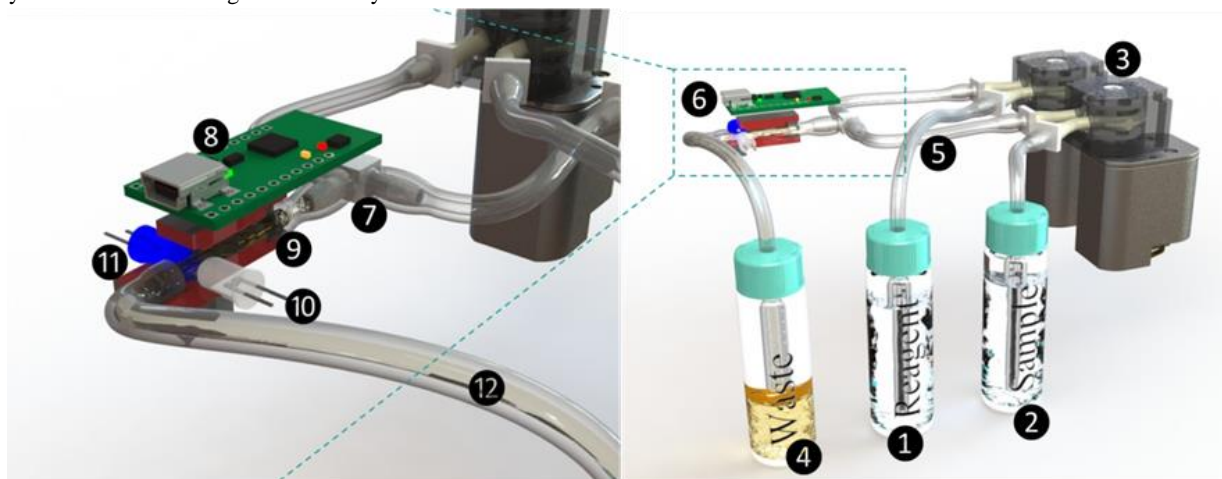


Fig. 4 Nitrate analyser and PEDD detection system. (1) Reagent storage (2) Sample storage (3) Peristaltic micro pumps containing Santropene® tubing (4) Waste storage (5) Tygon® tubing (6) PEDD flow cell (7) Mixing junction (8) Wixel microcontroller with breakout board containing wireless serial link and data logger (9) Glass flow cell (10) Detector LED at 630 nm (11) Emitter LED at 430 nm (12) Waste line (Tygon tubing).

2.4 Fabrication of PEDD optical detector and measurement procedure using nitrate analyser

The autonomous nitrate analyser manifold consisted of two peristaltic pumps, mixing junction, and a sensing channel in which the absorbance from the complex formed was measured using a PEDD system with a Wixel microcontroller (Pololu Corporation, USA).

The PEDD detector device contains two LEDs with one acting as the light source and the other acting as a detector as described previously. The LEDs were then placed into a PEDD flow cell generated by a 3D printer (Dimension SST 768) ensuring accurate alignment of the two LEDs.¹⁷

The fabrication of the PEDD cell was described in detail by O'Toole et al. and is illustrated in figure 3.¹⁷ Briefly, a regulated voltage source was used to power the emitter LED. A 430 nm LED (Radionics Ltd, Ireland) was used with a λ_{max} of 430 nm and an emission bandwidth at $1/2\lambda_{\text{max}}$ of 60 nm (Kingbright Specifications) which overlaps the absorbance spectrum of the nitrate—chromotropic acid complex. A variable resistor altered the LED light intensity while a transistor switched the LED on. The detector LED, connected in reverse bias (acting as a capacitor) to the microcontroller switchable input/output (I/O) pin, was supplied with 3.3 V for 100 μs and then switched to input mode. The detector LED generates a photocurrent related to the intensity of the light arriving from the emitter LED through the sample, and discharges this (3.3V) stored voltage. The more light absorbed by the sample, the slower the rate of discharge. The micro-controller then measures the time taken for the detector LED to discharge, in other words, the time taken to discharge from an initial value of 3.3 V (logic 1) to a preset value of 1.7 V (logic 0). This discharge time is referred to as the PEDD count.

The nitrate analyser manifold (figure 4) consists of two micro peristaltic pumps (Series 100, Williamson Manufacturing Company Ltd) employing Santoprene™ tubing of 3.2mm bore (Radionics Ltd, Ireland). The pumps deliver the sample and reagent via Tygon® medical grade tubing (formula 2075 1.D 1.6mm, Sigma Aldrich, Ireland) to the mixing junction. The coloured complex formed passes through the PEDD system in a modified glass flow cell (Brand Ltd Cat. No. 7477 15) at a combined flow rate of 0.8 ml/min and an optical path length of 2.5 mm.

The reagent and samples were pumped through the mixing chamber followed by a 15 minute reaction time prior to monitoring of the absorbance by the PEDD detector. This results in a minimum sampling frequency of 30 minutes due to the pumping of reagent and sample (5 minutes respectively) followed by reaction time (15 minutes) and detection (5 minutes). The detector signal was transmitted wirelessly to a laptop via the Wixel's wireless communication function and saved as a text file followed by analysis in Microsoft Excel.

2.5 Fabrication of heating device

In order to investigate the effect of heat on the reaction, a heating device was constructed consisting of 6 x 2 W metal film resistors which were placed within a vial holder surrounded by insulation foam. A regulated 12 V voltage supply was used to power the heating device. By choosing a low resistance of 100 Ohms for each resistor, a high current was produced, resulting in the heating of each resistor.

3. Results and Discussion

3.1 Optimisation of method parameters

3.1.1 Calibration

The optimum wavelength was investigated by obtaining the absorbance spectrum of a chromotropic acid and 80 mg/L nitrate complex. The absorbance of the complex is quite broad, with the λ_{max} around 430 nm. This was consistent with literature values, which typically range from ca. 400 – 450 nm.^{14, 16, 22} A calibration plot was performed using a set of prepared nitrate standards from 0.9 - 80 mg/L in a 1:1 v/v sample to chromotropic reagent ratio (figure 5A).

The molar extinction coefficient (ϵ) was estimated to be $1.302 \times 10^3 \text{ L mol}^{-1} \text{ cm}^{-1}$ at 430 nm, with a correlation coefficient of 0.991 and an average R.S.D. ($n = 3$) of 2.2%. The linear range was also improved to 80 mg/L nitrate compared to previous methods cited with a linear range of 0 – 11 mg/L nitrate.¹⁵

3.1.2 Kinetic study

The rate of development of the nitrate chromotropic reagent

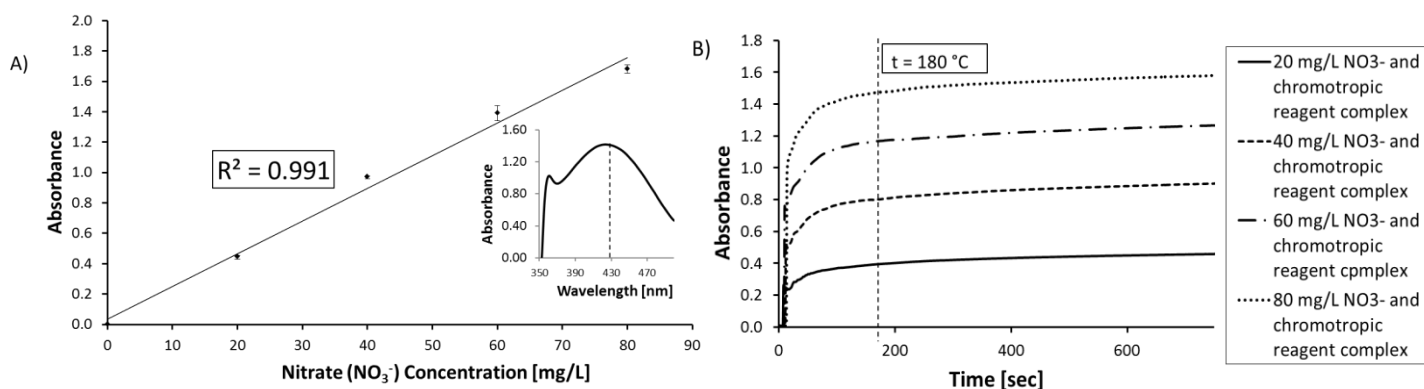


Fig. 5 (A) Calibration plot of nitrate standards ranging from 0 - 80 mg/L nitrate in the chromotropic acid reagent (1:1 v/v). The error bars represent the standard deviations for $n = 3$. The absorbance spectrum of the nitrate-chromotropic acid complex using 60 mg/L nitrate illustrating the λ_{max} at ca. 430nm is also displayed.(B) Kinetic study of the chromotropic acid-nitrate complex using various concentrations of nitrate [mg/L].

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complex colour intensity was monitored at a temperature of 23 °C for the detection of 20 – 80 mg/L nitrate. The absorbance at 430 nm was taken every 30 seconds for 900 seconds. The colour formation followed first order kinetics, increasing rapidly until approximately 180 seconds, after which the rate of increase was minimal as shown in figure 5 (B). Previously, a reaction time of 1800 seconds was recommended, but the significantly faster reaction time obtained here is an obvious improvement in terms of potential sample throughput in an autonomous integrated system.¹⁵

3.1.3 Limit of detection

The limit of detection (LOD) was determined by obtaining a calibration graph of low nitrate concentrations from 0.01 mg/L to 0.05 mg/L and the standard deviation (*SD*) of the baseline of the blank which consisted of the chromotropic acid reagent and 0 mg/L nitrate (*n*=60 data points, frequency of measurement: 1 data point/sec) figures for which are shown in table 1. The LOD was calculated as the concentration equivalent of 3 *SD* of the blank solution, giving a value of 0.73 µg/L nitrate, a significant improvement on the previously reported limit of detection of 0.12 mg/L nitrate.¹⁵ In practice however the LOD is limited to the concentration equivalent to the absorbance of the blank solution giving a LOD of approximately 0.64 mg/L nitrate.

3.2 Validation of Method

Following optimisation of the measurement parameters, a validation process was implemented using 8 samples from various environmental sources including waste water effluent, surface water, drinking water and standards. It was noted from the literature that only nitrite ions give a positive interference when determining nitrate with the proposed method.¹⁴ As nitrite in the micro molar region is found only in deep eutrophic levels and nitrite present in surface water is readily oxidised to nitrate, nitrite interferences were not of major concern.¹⁴ The samples were split, and parallel assays independently performed at the T.E. Laboratory site. The samples were filtered prior to analysis

Concentration	Average Absorbance	Standard Deviation
Blank	0.0032	0.00026
0.01	0.0106	0.00049
0.02	0.0228	0.00010
0.03	0.0316	0.00032
0.04	0.0398	0.00081
0.05	0.0552	0.00060

Table 1. Absorbance values of blank (0 mg/L nitrate and chromotropic acid reagent) and low nitrate concentrations (0.01 – 0.05 mg/L nitrate and chromotropic acid reagent.)

using nylon membrane filters with pore size of 0.45 µm (Acrodisc® syringe filters, Sigma, Ireland). The chromotropic method was used at both sites and the results compared to reference measurements obtained using ion chromatography.²³

There is a strong correlation as illustrated in figure 6 between the chromotropic method and ion chromatography concluding that the chromotropic method is ideal for integration into an autonomous analyser for the direct detection of nitrate. The discrepancy observed for sample E (nursing home effluent), with the chromotropic acid method showing a concentration of ca. 90 mg L⁻¹ nitrate compared to 115 mg L⁻¹ nitrate obtained using ion chromatography, is due to the sample concentration being above the linear range for the chromotropic acid method as described above.

3.3 Limitations of method

It should be noted that difficulties may arise when mixing the sample and chromotropic reagent. As mentioned previously the heat energy produced from the exothermic reaction, due to the dilution of the concentrated sulphuric acid, aids the colour formation for the colorimetric detection of nitrate. It was observed at the benchtop level, that significant discrepancies can be found when varying the mixing and addition techniques of the sample to the acidic reagent. These discrepancies may be due to different mixing behaviour arising from the manual addition of the reagents to the cuvette, which leads to differences in the quantities of heat energy released, which in turn affects the formation of the coloured complex. For instance, if the sample is added rapidly the acid will react with the water more vigorously, generating a higher temperature, and creating an intense colour. On the contrary, if the sample is added more gradually to the reagent, the heat energy is developed over a longer time, resulting in a less intense colour.

Notwithstanding these issues, by implementing the method into an integrated flow analysis platform, these inconsistencies are significantly reduced by consistent pumping of reagent and sample and reproducible mixing behaviour in the flow system.

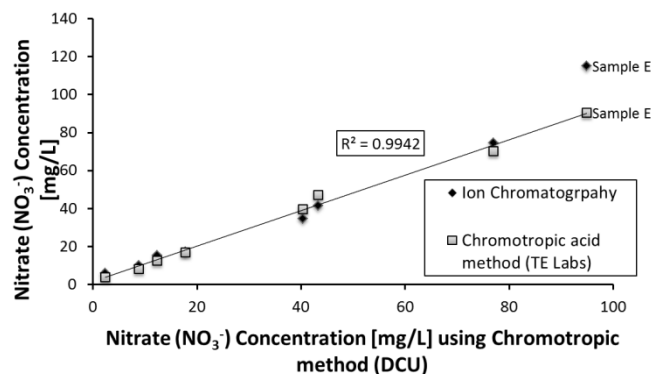


Fig.6 Correlation plot of sample concentrations obtained using the chromotropic acid method (DCU) and ion chromatography (TE Labs).

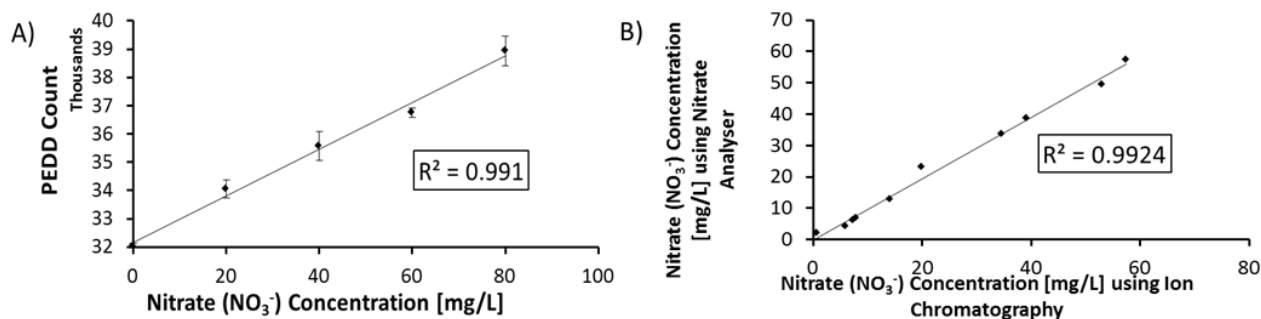


Fig. 7 (A) Calibration curve using the nitrate analyser from 0 - 80 mg/L nitrate and chromotropic acid reagent complex. The standard deviations as represented as error bars. (B) Correlation plot of sample concentration obtained by nitrate analyser and ion chromatography.

4. Integration of method into flow analysis platform

The chromotropic reagent and a range of standards were prepared as per section 2. Figure 7 (A) shows the calibration plot obtained using nitrate standards up to 80 mg/L with an average R.S.D. (n = 3) of 0.9%.

10 blind samples were provided by T.E. Laboratories Ltd. and analysed with the prototype nitrate analyser platform. In order to provide reference data, the samples were also measured using ion chromatography. The strong correlation between the nitrate analyser and ion chromatography is shown in figure 7 (B).

5. Effect of sulphuric acid concentration and heat on nitrate-chromotropic acid complex formation

An area of concern that has been highlighted with this method is the use of 96% sulphuric acid (ca. 18 M) that must be present in the chromotropic acid reagent for effective formation of the nitrate complex. This is an issue as the strongly acidic environment drastically constrains the materials that can be used to store and move the reagent in the overall fluidic system.

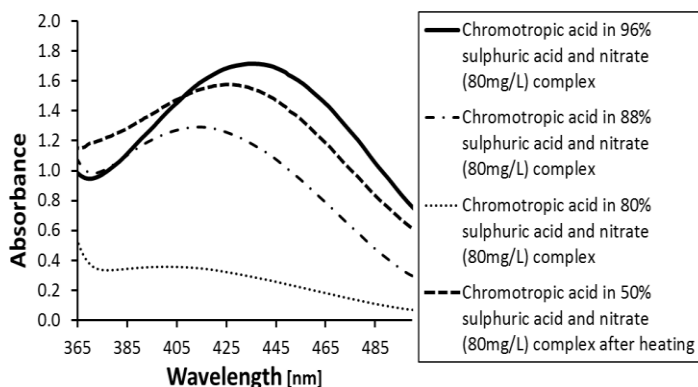


Fig 8. Spectra of 80 mg/L nitrate and chromotropic acid with varying concentrations of sulphuric acid within the chromotropic acid reagent.

Therefore it is of interest to reduce the sulphuric acid concentration. The effect of reducing the sulphuric acid concentration was therefore investigated by obtaining an absorbance spectrum of a mixture of 80 mg/L nitrate and the chromotropic acid reagent, while reducing the background concentration of the sulphuric acid from 96% - 80% (v/v).

The results (figure 8) show that lowering the concentration of the sulphuric acid below 96%, leads to a large reduction in the absorbance. However, reducing the acid concentration also reduces the spontaneous heat generated by mixing of the reagent with the sample (i.e. these results were obtained without external heating).

The chromotropic acid reagent was prepared as per section 2.2 with the exception of using 50% sulphuric acid in the reagent. To this, the 80 mg/L nitrate sample was mixed in a 1:1 volume ratio and then heated in the custom built heating device described in section 2.5, in a 4 ml capped vial for 25 minutes until a temperature of 130 °C was achieved as measured using a laser infrared heat thermometer (Walyou Ltd).

The spectrum presented in figure 8 shows that strong colour formation can be produced despite significantly decreasing the sulphuric acid concentration by introducing external heating. The blank consisted of the chromotropic acid reagent and 0 mg/L nitrate. The absorbance value obtained using the 50% sulphuric acid reagent is similar to the absorbance value achieved using the 96% sulphuric acid reagent. These preliminary results are significant as the extremely high concentration of sulphuric acid recommended for the method can be avoided, which simplifies the integration of the method into an autonomous flow system, and broadens the range of possible materials that can be used.

6. Conclusion

An analysis system for the direct determination of nitrate in water using chromotropic acid has been developed. The chromotropic acid method has been modified to facilitate its implementation into a flow analysis platform, resulting in a quick and simple procedure to measure nitrate. The results presented here are the first for a nitrate analyser incorporating the chromotropic acid method and the PEDD detector. This forms the basis of a simple and low cost autonomous detection device which could be used as an alternative to expensive optical detectors for the detection of nitrate in water. This study demonstrates advantages including

the low limit of detection of 0.73 µg/L, the wide linear range of 0.9 – 80 mg/L, and the fast response time of the method. The flow analysis system possesses a sample turnaround of 30 minutes, which may be capable of significant further improvement through further operational optimisation. It was also proved within this study that the sulphuric acid concentration could be halved while maintaining satisfactory analytical results producing a safer and more convenient method. The device incorporates low cost, highly sensitive detection with excellent correlation to the standard method, ion chromatography. Ultimately, this system may provide a base for monitoring waters for nitrate levels in situ in a rapid, simple and inexpensive manner.

7. Acknowledgments

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