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Determination of nitrite by inhibition of the chemiluminescence of acriflavine in a flow-injection assembly

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The indirect determination of nitrite was performed with a flow-injection assembly on the basis of the inhibition of the analytical output obtained in a luminometer by oxidation of acriflavine. The acriflavine solution merged with the nitrite and the resulting mixture was injected into a pure water stream. This solution merged with the oxidant solution (potassium permanganate in sulfuric acid medium) and the resulting chemiluminiscence was affected (inhibited) by the presence of nitrite after reaction with the aminoacridine. The method was applicable over the range 10–800 μg l^{-1} of nitrite with a correlation coefficient of 0.9960. The relative standard deviation was 1.4% and the throughput was 76 samples h^{-1}. The influence of foreign substances was also tested. A solid-phase reactor, filled with Amberlite IRA-900, was inserted in the assembly for the on-line preconcentration of nitrite; the analytical output resulted in an increase of up to 11.5-fold. The method was applied to the determination of nitrites in residual waters, industrial formulations and soil samples.

Introduction

The determination of nitrite in various types of samples, particularly environmental samples and foods, is growing in significance as a result of the increasing eutrophication of natural waters and salinisation of aquifers, as well as the health problems caused by cyanosis and the formation of nitrosamines in living organisms from amines and nitrites used as food additives. Nitrite levels are routinely determined in quality control analyses of drinking, waste, marine and underground waters, among others. This has aroused an interest in developing new analytical methods for determining nitrite in all types of samples. The most common choice for this purpose continues to be the spectrophotometric method based on the Griess reaction, which has been the subject of several modifications. Alternative spectrophotometric reagents have also been used for the same purpose.

The number of articles devoted to nitrite determination in different kinds of samples is large and a number of instrumental procedures have been described. The nitrite–chemiluminescence combination has received the attention of about 50 articles (from 1980); however, in spite of the relatively large number of papers very few chemical reactions have been exploited. The largest number of papers are devoted to developing the emission of light from luminol, for example, reaction of nitrite with iodide followed by reaction of the produced iodine with luminol,[1] oxidation of nitrite with hydrogen peroxide and reaction of the generated peroxynitrite anion with luminol,[2] a nitrate–luminol photochemical reaction,[3] the hexacyanoferrate–luminol system,[4] and immobilised luminol.[5] Another widely exploited chemiluminescent system is the reaction with ozone as a gas-phase chemiluminescence reaction, either directly for gas samples or for others after volatilization for separation from the sample matrix.[6–11] Other published chemiluminescent sytems include hydrobromic acid,[12] and hydrogen peroxide–bis(2,4,6-trichlorophenyl) oxalate.[13] In two papers the produced chemiluminescence was detected by a thermal energy detector.[14,15]

Acridines are anthracene derivatives. Acridine (the parent member of the family) is an anthracene molecule containing a nitrogen atom in the central ring. The acridine family is very large and includes diaminoacridines such as proflavine and acriflavine. These aminoacridines react with nitrite; the reaction with proflavine was first reported in the early 1900s[16] but was not studied until much later.[17] The violet colour that develops in the reaction was ascribed to the formation of a quinoneimine, which, in the presence of nitrous acid, yields the bis-diazo derivative to some extent. This reaction was used in conjunction with spectrophotometric detection for the determination of nitrite with proflavine, both in a static system[18] and in continuous-flow mode by means of a flow-injection analysis (FIA)[19] or a tandem-flow assembly.[20]

On the other hand, we have described the positive chemiluminescent behaviour of some members of the diaminoacridine family when oxidized by strong inorganic oxidants.[21]

This paper reports a method for the determination of nitrite in water, soil and industrial samples based on the chemiluminescence of acriflavine (proflavine behaves identically) on oxidation with potassium permanganate in a sulfuric acid medium. The presence of nitrite and its previous reaction with the diaminoacridine decreases the luminescence intensity, and this inhibitory effect on the native chemiluminescence of acriflavine is used to determine the nitrite ion.

Experimental

Reagents

All reagents were of analytical-reagent grade unless stated otherwise. Aqueous solutions were prepared in de-ionized water (18 MΩ cm) obtained by use of a Sybron/Barnstead Nanopure II water-purification system provided with a filter of 0.2 μm pore size. Acriflavine (from Aldrich-Europe), $KMnO_4$ (Panreac), and sulfuric and hydrochloric acids (Merck) were used. The following reagents were also employed (all from Probus): $NaNO_3$, $Cr(NO_3)_3{\cdot}9H_2O$, $Fe(NO_3)_3{\cdot}9H_2O$, $Zn(NO_3)_2{\cdot}6H_2O$,

$Na_2HPO_4·12H_2O$, $Ca(NO_3)_2·3H_2O$, $Mn(NO_3)_2·6H_2O$, sodium dodecyl sulfate (SDS) and Na_2EDTA. Other reagents used (all from Panreac) were: $Na_2SO_4·10H_2O$, sodium acetate trihydrate, $Na_2CO_3·10H_2O$, $Al(NO_3)_3·9H_2O$, $Co(NO_3)_2·6H_2O$, $Pb(NO_3)_2$, $Ni(NO_3)_2·6H_2O$, $Cu(NO_3)_2·3H_2O$, KNO_3, NH_4NO_3 and NaF. In addition, $Cd(NO_3)_2·4H_2O$ (UCB), $NaHCO_3$ (Guinama), NaI (Scharlau), $HgCl_2$, $AgNO_3$ and $Mg(NO_3)_2·6H_2O$ (Prolabo) and phenol (Doesder) were also used.

Flow-injection (FI) assembly

The proposed FI manifold is depicted in Fig. 1. The flow manifold consisted of a PTFE coil of 0.8 mm id, a Rheodyne 5041 injection valve, and a Gilson Minipuls 2 peristaltic pump. Chemiluminescence measurements were performed by means of a home-made flow cell which consisted of a flat spiral-coiled quartz tube (1.0 mm id, 3 cm total diameter, without gaps between loops). The flow cell was placed about 2 mm from the photomultiplier tube window (Thorn-EMI) and backed by a mirror for maximum light collection. The flow cell and photomultiplier tube were placed inside a home-made light-tight box. The photomultiplier was operated at 1273 V supplied by a PHV-40 programmable high voltage power supply (Acton Research). Spectrophotometric measurements for the reference method were performed with a Hewlett-Packard diode-array spectrophotometer (Model 8452) provided with a flow cell of 18 μl inner volume from Hellma.

Optimisation of the flow assembly; chemical and hydrodynamic parameters

The optimisation of the chemical and FIA variables was performed by means of a sequential combined methodology. First, the chemical parameters were optimised; then, with the selected chemical values, we optimised the FIA hydrodynamic variables by using a multivariate method, *viz*., the modified simplex method (MSM).

The initial simplex was selected according to Yarbro and Deming.[22] The variable region was standardised by following the modification of Morgan and Deming.[23] The different simplex vertices were obtained with the aid of software based on the method of Nelder and Mead[24] with the target FIA variables and the value (in μA) corresponding to each combination of such variables provided by the simplex inputs. The program was written to optimise the height of the output. Two consecutive simplex operations were performed, the interval for each variable in the second being restricted to the zone that gave the best results in the first. Then, we selected some of the higher vertices for a new comparative study to choose the output resulting in the best compromise sensitivity (peak height)–sample throughput (peak-base width)–reproducibility (RSD, %).

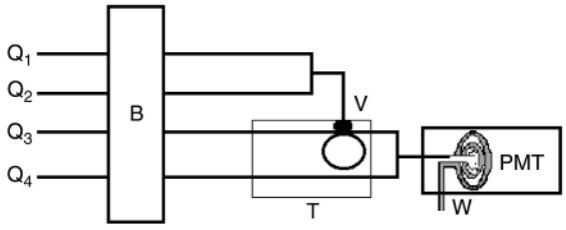

Fig. 1 Flow-injection assembly for chemiluminiscent determination of nitrite. Q_1/Q_2: nitrite/acriflavine (1:5); Q_3: de-ionized water (5 ml min^{-1}); Q_4: 3×10^{-4} mol l^{-1} potassium permanganate in 0.1 mol l^{-1} sulfuric acid (1 ml min^{-1}); sample loop: 647 μl; T: thermostated bath at 60 °C; B: peristaltic pump; V: injection valve; PMT: photomultiplier tube; W: waste.

Preparation of the samples

The sample of residual water was filtered and diluted to the corresponding nitrite concentration. The industrial formulation only required to be weighed, dissolved and diluted to a suitable concentration. A 75 g amount of the soil sample was stirred with 150 ml of water for 30 min; the mixture was then filtered and the filtrate was made up to 250 ml.

Results and discussion

Preliminary tests

Four members of the acridine family were initially studied, *viz*. acriflavine, acridine, proflavine and Acridine Yellow, all in an assembly similar to that of Fig. 1. Channel Q_3 was used to circulate the carrier (a 0.1 mol l^{-1} stream of sulfuric acid), into which a solution of the specific acridine at a 1.36×10^{-5} mol l^{-1} concentration was inserted. Q_4 was used to circulate the oxidant stream (6.0×10^{-5} mol l^{-1} potassium permanganate in 0.1 mol l^{-1} sulfuric acid). This stream and Q_3 were merged at a point near the luminometer flow cell. Chemiluminescence signals for the acridines were obtained in both the presence (inhibited signals) and absence of nitrite. A calibration graph run from 0.08 to 0.8 mg l^{-1} revealed that the signal was inhibited to a much greater extent for acriflavine than for the other acridines, so the former was chosen for subsequent experiments (see Fig. 2). The chemical features of the FIA system were altered as shown in Table 1 and a calibration graph for acriflavine over the range 2–20 mg l^{-1} was obtained in order to determine the linear range, over which calibrations for nitrite using acriflavine concentrations of 2, 4, 6 and 8 mg l^{-1} were performed. The working range expanded with increasing concentration of acriflavine, albeit at the expense of a decreasing sensitivity to nitrite. An acriflavine concentration of 6 mg l^{-1} was adopted as the best compromise.

Influence of physico-chemical variables

In a subsequent experiment, the sulfuric acid concentration used in the acriflavine stream was varied from 0 to 0.2 mol l^{-1}. Although the acid increased the signal yielded by acriflavine, it also decreased the inhibitory effect of low concentrations of

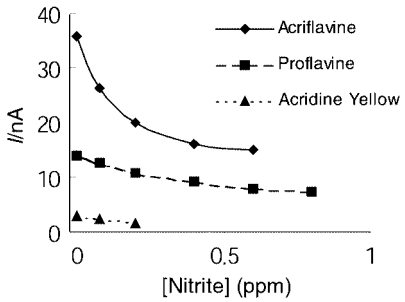

Fig. 2 Influence of the nitrite concentration on the chemiluminescent output. The acridine signal was zero.

Table 1 Parameters of the flow assembly for preliminary assays

Variable			
Q_1/ml min^{-1}	1.4	Carrier	Water
Q_2/ml min^{-1}	1.4	Oxidant	MnO_4^-, 3×10^{-4} M
Q_3/ml min^{-1}	3.4		H_2SO_4, 0.05 M
Q_4/ml min^{-1}	1.0		
V/μl	400		

nitrite, probably through decomposition of the ion. For this reason, the acriflavine solution was prepared in water instead of the acid.

The concentration of sulfuric acid in the oxidant was varied over the range 0.025–0.3 mol l^{-1}; 0.2 mol l^{-1} was found to provide the best compromise between selectivity and working range (see Fig. 3). Similarly, the effect of the permanganate concentration was studied between 2×10^{-4} and 4×10^{-4} mol l^{-1}, and 3×10^{-4} mol l^{-1} was chosen as the most suitable.

Temperature exerts a marked effect on the chemiluminescence of acriflavine. In order to examine its influence, the PTFE tubing used to circulate the oxidant solution (1 m long) and the water carrier (1 m long prior to the injection valve) was immersed in a thermostated bath at temperatures from ambient to 80 °C, as was the loop of the injection valve. Raising the temperature was found to increase the signal but also to decrease repeatability (see Fig. 4). For convenience, a temperature of 40 °C was chosen with a view to its subsequent re-optimisation.

At this point, the influence of the presence of a small amount of acid in the acriflavine solution was re-assessed. To this end, acriflavine solutions containing different concentrations of HCl (0.01–0.03 mol l^{-1}) or H_2SO_4 (0.01–0.05 mol l^{-1}) were tested. No appreciable effect on the signal was observed, so the acriflavine solution continued to be prepared in water.

Optimisation of hydrodynamic parameters

The univariate method was used to optimise the acriflavine : nitrite flow-rate ratio. First, the acriflavine flow rate was kept constant at 1.4 ml min^{-1} and the nitrite flow rate was changed from 1.2 to 3.9 ml min^{-1}. The best results were obtained with a nitrite flow rate of 3 ml min^{-1}, which was then kept constant while a 10 mg ml^{-1} acriflavine solution was circulated at flow-rates between 0.6 and 2.3 ml min^{-1}. The best acriflavine flow-rate was 0.6 ml min^{-1}, so the optimum acriflavine : nitrite flow rate ratio was 1 : 5.

Following re-optimisation of the acriflavine concentration between 10 and 40 mg l^{-1}, which yielded a new optimum value of 25 mg ml^{-1}, the FIA variables were optimised using the modified simplex multivariate method. The input variables used were the carrier and oxidant flow rates, and the injected volume—the length of tubing for the carrier stream behind the injection valve was minimised in order to avoid dispersion of the sample. The ranges over which the three variables were studied and their optimum values are shown in Table 2.

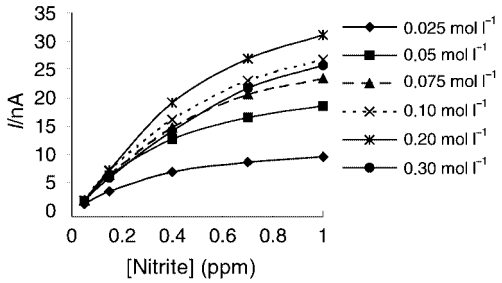

Fig. 3 Effect of the sulfuric acid concentration in the oxidant solution on the chemiluminescent inhibition.

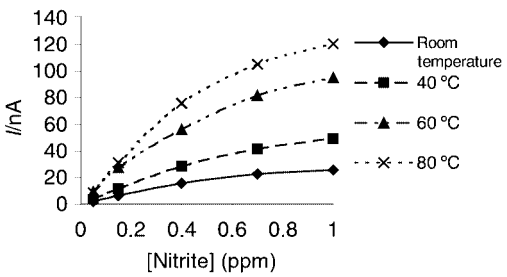

Fig. 4 Effect of the temperature on the chemiluminiscence output.

Re-optimisation of physico-chemical variables

The optimum FIA assembly was used to re-optimise the physico-chemical variables. First, the effect of temperature was studied over the range 40–70 °C. Increasing the temperature was found to increase both the blank signal (acriflavine) and the inhibitory effect of nitrite. However, it also decreased the reproducibility, particularly above 60 °C, which was thus the chosen temperature. The sulfuric acid concentration was re-optimised between 0.05 and 0.3 mol l^{-1}, and 0.1 mol l^{-1} was adopted as optimum. Finally, the concentration of permanganate was kept at 3×10^{-4} mol l^{-1} (following testing between 2×10^{-4} and 4×10^{-4} mol l^{-1}) and that of acriflavine at 25 mg l^{-1} (the most suitable value in the range 15–36 mg l^{-1}).

The influence of various surfactants and sensitizers with known effects on chemiluminescent reactions was studied. The substances tested included SDS and hexadecylpyridinium chloride (both at a 1.5% concentration); Triton X-100 and β-cyclodextrins (both at a 0.5% content); and quinine sulfate, 8-hydroxyquinoline, Acridine Orange, Rhodamine B and Rhodamine 6G (all at a 5×10^{-4} mol l^{-1} concentration). Although some of these substances were found to enhance the chemiluminescence of acriflavine, the influence on the inhibitory effect of a 0.3 μg ml^{-1} solution of nitrite was negative or zero in all cases.

Influence of acriflavine composition

Acriflavine is a mixture of 3,6-diamino-10-methylacridinium chloride hydrochloride and proflavin. However, the composition of this mixture is well established and includes 30–40% of the unmethylated compound proflavin. Owing to the different contents of both proflavin and the methylated compound the influence of the composition was examined. Acriflavine from Aldrich, Guinama and Fluka was tested at two different concentrations of nitrite (100 and 550 μg l^{-1}) using the optimised manifold depicted in Fig. 1 and a solution containing 50 mg l^{-1} of acriflavine. The inhibitory analytical signal [(acriflavine + nitrite) − (acriflavine)] was not markedly affected by slight changes in the composition of acriflavine. The RSD values obtained for the inhibitory response of the three acriflavines tested were 8.5 and 7% for 100 and 550 μg l^{-1} of nitrite, respectively.

Analytical figures of merit

The limit of detection, calculated as the lowest nitrite concentration inhibiting the analytical signal to an extent more than three times greater than the absolute standard deviation of the blank signal, was 10 μg l^{-1}.

The linear range for nitrite was 10–800 μg l^{-1}, over which the analytical signal fitted a second-order polynomial equation (or a straight line in a log–log plot). The average equation for eight

Table 2 Optimisation of the hydrodynamic variables by the multivariate modified simplex method

Variable	Simplex 1	Simplex 2	Optimum
Carrier flow rate/ml min^{-1}	1.2–5.7	4.2–5.7	5
Oxidant flow rate/ml min^{-1}	0.6–2.6	0.7–1.9	1
Sample volume/μl	48–637	441–932	647

curves obtained using fresh solutions on different days was

$$\log \Delta I = (0.70 \pm 0.03) \log C + (2.48 \pm 0.05) \quad (r = 0.996)$$

where the intensity inhibition (ΔI) is expressed in nA and the concentration of nitrite ion (C) in mg l^{-1}.

The RSD for a series of 30 injections of a 0.35 μg ml^{-1} solution of nitrite was 1.4%. The throughput, calculated using the same series, was 76 samples h^{-1}.

The influence of foreign species accompanying nitrite in its samples (*viz*. metal ions, common inorganic ions and organic compounds) was studied using solutions containing 350 μg ml^{-1} nitrite and decreasing concentrations of each foreign species (from an initial level of 500 mg ml^{-1} in most cases). The signals thus obtained were compared with that provided by a pure nitrite solution in order to determine the relative errors resulting from the presence of the foreign species (see Table 3).

Various samples containing nitrite, which were prepared as described under Experimental, were analysed using the proposed method and the Griess method (as implemented in an FIA system). The results and the errors relative to the official method are compared in Table 4.

Determination of nitrite by preconcentration on Amberlite IRA-900 resin

In order to improve the detection limit, a preconcentration column was inserted into the above-described FIA assembly.

Table 3 Study of interferences

Interferent	Concentration/ mg l^{-1}	Relative error (%)
Nitrate	1000	1.9
Chloride	725	2.4
Acetate	200	3.0
Hydrogencarbonate	500	2.7
Phosphate	400	1.5
Sulfate	500	0.8
Fluoride	100	2.0
Iodide	0.15	2.2
Ammonium	400	2.3
Copper(II)	4.0	3.0
Nickel(II)	300	2.8
Cadmium(II)	270	0.4
Lead(II)	20	1.6
Iron(III)	8	2.8
Sodium(I)	470	2.4
Potassium(I)	848	2.4
Magnesium (II)	250	2.1
Silver(I)	500	1.9
Manganese(II)	20	1.3
Aluminium(III)	200	2.1
Cobalt(II)	30	3.0
Mercury(II)	500	2.6
Zinc(II)	500	0.2
Barium(II)	500	1.6
Calcium(II)	500	2.6
Chromium(III)	60	1.4
Na_2EDTA	20	2.0
Sodium dodecyl sulfate	40	3.0
Hexadecylpyridinium chloride	100	1.2
Urea	1000	2.1
Phenol	1.5	0.4

Table 4 Analysis of real samples

Sample	FIA method	Reference method	Relative error (%)
Residual water/μg ml^{-1}	0.75	0.73	2.7
Industrial formulation (%)	4.50	4.42	1.8
Soil/μg g^{-1}	62.2	61.1	1.8

The reactor was constructed by packing a piece of PTFE tubing (17 cm × 3.9 mm id) with Amberlite IRA-900 anionic resin of particle size 300–1180 μm. The reactor dimensions were substantially greater than those used in similar preconcentration processes, a necessary condition in order to reproduce as accurately as possible the concentration profile or gradient obtained in the absence of the reactor—the concentration gradient obtained following elution of retained nitrite should be as close as possible to that produced by the injection of 647 μl of the nitrite–acriflavine mixture. Unlike other spectrophotometric and fluorimetric procedures, the flow cells used in chemiluminometers possess large volumes and lengths in order to collect as much emitted light as possible, so the increased dispersion must be countered by using increased sample volumes.

For this experiment, the optimum FIA assembly was modified with two new injection valves, the functioning of which is depicted in Fig. 5. Each peak, corresponding to one cycle, was obtained by preconcentrating nitrite for a variable time, flushing the reactor with de-ionized water for 30 s, eluting the retained nitrite with 0.02 mol l^{-1} HCl until the baseline was restored and, finally, flushing with de-ionized water for 60 s before the next cycle was started. The best concentration gradient for a nitrite–acriflavine mixture was found to be that obtained when the mixture was injected 70 s after elution had been started.

The preconcentration factor was determined by passing variable volumes of sample through the column, using a constant flow rate of 5 ml min^{-1} for variable lengths of time. The inhibitory effects on the chemiluminescence of acriflavine produced by different volumes of sample are shown in Table 5. The first column (ΔI) indicates the analytical signal in nA yielded by passing through the column of the manifold depicted in Fig. 5 different volumes of an aqueous solution containing 3 and 10 μg l^{-1} of nitrite. The analytical outputs were obtained by operating as described in the previous paragraph. The second column ($[NO_2^-]$ recovered/μg l^{-1}) refers to the concentration of nitrite that would yield an identical signal in the manifold of Fig. 1 (in the absence of preconcentration). These values were

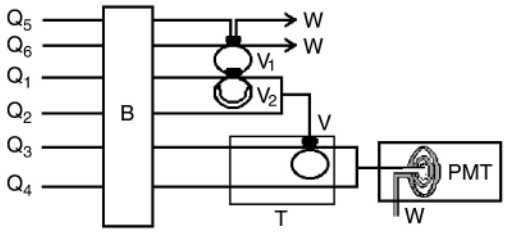

Fig. 5 Flow injection assembly provided with an Amberlite IRA-900 exchange reactor for nitrite preconcentration. The obtention of a peak was performed as follows: (1) preconcentration step: aqueous nitrite solution flowing at 5 ml min^{-1} through the column; V_1 and V_2 in load position; t variable. (2) Washing step: de-ionized water flowing at 4 ml min^{-1}; V_1 in injection and V_2 in load position; 30 s. (3) Elution step: 0.02 M HCl until baseline was restored; V_1 and V_2 in injection position. (4) Washing step: de-ionized water flowing at 4 ml min^{-1}; V_1 and V_2 as in step (2); 60 s.

Table 5 Pre-concentration of nitrite using an Amberlite-900 exchange reactor

	$[NO_2^-]$ = 10 μg l^{-1}		$[NO_2^-]$ = 3 μg l^{-1}	
Volume to be preconcentrated/ml	ΔI/nA	$[NO_2^-]$ recovered/ μg l^{-1}	ΔI/nA	$[NO_2^-]$ recovered/ μg l^{-1}
25	28.5	34	13.6	12.5
40	35.6	47	15.3	14.6
55	46.8	68	18.7	19.3
70	53.8	82	24.1	27.3
90	61.9	100	28.5	34.4

calculated from the double logarithmic calibration equation obtained by injecting nitrite solution over the linear range 10–800 μg l^{-1}.

Conclusions

A straightforward, expeditious method for the determination of nitrite in water, soil and industrial samples is proposed, the selectivity of which relies on the inhibition of the chemiluminescence of acriflavine, a member of the acridine family, through its coupling reaction with nitrite. The use of an Amberlite IRA-900 column to preconcentrate nitrite allows its determination at levels that make the proposed method competitive with classical alternatives based on the Griess reaction or the more recent luminol reaction. The equipment used, which possesses no moving or fragile optical parts, is robust, simple and affordable.

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