



Tetrazine-based chemistry for nitrite determination in a paper microfluidic device



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ABSTRACT

We present a new chemistry to determine nitrites implemented in a microfluidic paper-based analytical device (μ PAD). The device is fabricated in cellulose paper with a sample reception area and three replicate detection areas with recognition chemistry immobilized by adsorption. The method involves the use of nitrite in an acid medium reaction to generate nitrous acid, which produces the oxidation of *s*-dihydro-tetrazine: 1,2-dihydro-3,6-bis(3,5-dimethyl-1H-pyrazol-1-yl)-1,2,4,5-tetrazine (DHBPTz), which change the detection zone from colorless to pink. We used a digital camera and smartphone for the quantitative analysis of nitrite with the color coordinate *S* of the HSV color space as the analytical parameter. Parameters such as concentration and volume of *s*-dihydro-tetrazine, pH, sample volume and reaction time were studied. The detection limit for this method is 1.30 μ M nitrite. To estimate the selectivity of the method an interference study of common ions in water samples was performed. The procedure was applied to natural water and compared with reference procedures.

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1. Introduction

Nitrite is a ubiquitous and naturally occurring species that plays an important role in different physiological, food, environmental and industrial systems. Different approaches have been used for analysis, with the most common being spectrophotometric, luminescent, potentiometric, amperometric, and separative procedures [1–3]. Most spectrophotometric methods use the Griess reaction based on azo dye formation [4] in different variants because it offers the right balance between simplicity, instrumental needs, cost and sensitivity [5]. These methods, however, have several drawbacks such as the need to control the pH, the temperature, the use of multiple reagents, some of them carcinogenic, and a long reaction time [6].

To overcome some of these disadvantages, we have developed a user-friendly paper-based device based on a new *s*-tetrazine-based chemistry for nitrite determination that is being presented for the first time, to the best of our knowledge.

s-Tetrazines, a class of high-nitrogen organic compounds, have received much synthetic attention because of their inherent

biological potential [7,8]. They have also been frequently used as building blocks in organic transformations or as bridging ligands in metal complexes [8–10]. In particular, 1,2,4,5-tetrazines [11,12] are the most important class of tetrazines that, due to the four electronegative nitrogen atoms in the tetrazine ring, have great synthetic utility and are capable of participating in strain-promoted inverse-electron-demand Diels–Alder cycloaddition reactions with an applicability in bioconjugation [13–15]. They also provide access to a wide range of other heterocycles such as dihydropyridazine and pyridazine systems [16].

In the actual state-of-the-art, the *s*-tetrazine ring is always obtained indirectly by oxidation of its 1,2- or 1,4-dihydro (or even in rare cases its tetrahydro) counterpart, which can be prepared by various methods. Five main different strategies have been reported to date [17], but the long used Pinner synthesis and a modified version of it [18,19] are still the most commonly used. The Pinner synthesis involves the reaction of hydrazine with a nitrile, always leading to a dihydro-tetrazine that has to be further oxidized into the fully aromatic tetrazine. An versatile alternative strategy is that introduced by Scott based on the preparation of 1,2-dihydro-3,6-bis(3,5-dimethyl-1H-pyrazol-1-yl)-1,2,4,5-tetrazine (DHBPTz) [20]. This heterocycle is readily prepared via a condensation reaction of a triaminoguanidinium salt and acetylacetone in water [21–24].

Analytical applications of tetrazines are few and derived from

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three characteristics of their most stable form (1,2,4,5 isomer; s-tetrazines): a) the strong electron deficient character of the ring which leads it to accept electrons and protons simultaneously, producing dihydrotetrazines via anion radical [7,25]; b) the low-lying π^* orbital resulting in highly colored compounds, typically red, due to an $n\text{-}\pi^*$ transition in the visible light; additionally, some tetrazines, namely those replaced by electronegative heteroatoms (F, Cl, O) directly on the ring, show fluorescence both in solution and in the solid state with medium quantum yield ($\Phi_f < 0.4$) and a lifetime typically in the 10–160 ns range in solution [9,23,26–28]; c) the presence of at least four nitrogen donor atoms at the molecule that result in a coordination ability characterized by electron and charge transfer phenomena and efficient metal-metal bridging capacity [9,29].

Chung et al. identified gram-positive bacteria using a two-step labeling procedure by reacting by cycloaddition, nanoparticles labeled with a fluorescent tetrazine with bacteria bonded to the bacterial cell wall with vancomycin or daptomycin derivatives [30].

A colorimetric sensing material for VOCs has been developed based on a nanotubular metal-organic framework (MOF) including 3,6-di(pyridin-4-yl)-1,2,4,5-tetrazine and $\text{WS}_4\text{Cu}_4^{2+}$ units. The combination of a sieving function along with solvatochromism makes it possible to discriminate small solvent molecules, both liquid and vapors, which is ascribed to its strong π -acceptor property and labile electronic structure to solvent polarity [31].

The strong electron-deficient character of tetrazines is more prominent in their first excited state, which means that fluorescent tetrazines can be quenched by electron donors [7]. The quenching that occurs between the tetrazine excited state and the donor through electron transfer has been proposed for the determination of different protic and aprotic compounds in solution such as amines, PAH, phenols, etc. [26,32]. Malinge et al. used the extinction of fluorescence by the photoinduced electron transfer of silica nanoparticles functionalized with trialkoxysilanetetrazines for low-molecular weight amine sensing [28].

There are few examples of the use of tetrazines as chromogenic reagents for metal ions, the earliest being the paper by Schilt et al. on ferriin-type reagents for Fe(II), Cu(II) and Co(II) that include some pyridyltetrazines [33]. Samanta et al. [34] used pyridyltetrazines for the selective determination of Cu(II), Ni(II) and Ag(I) in aqueous solution through a different approach that increases their solubility in water through a facile synthesis of tetrazine capped silver nanoparticles. Complexation of metal ions by tetrazine moieties from adjacent NPs causes aggregation shifting in the plasmon band energy at longer wavelengths, making it possible to determine the metal ions both in solution and in test paper format. The coordination ability of tetrazines has been used by Roushani et al. to develop a polyacrylic ion imprinted polymer including DHBPTz moieties for extraction and the pre-concentration of Fe(III) [35].

The change in color of dihydro-tetrazines by oxidation has been proposed only once to the best of our knowledge for sensing of oxidant gases. The dihydro-1,2,4,5-tetrazine-3,6-dicarboxylate was introduced into a chemically stable UiO-66-based MOF structure by a post-synthetic linker exchange reaction [36]. The tetrazine functionalized zirconium MOF can be oxidized by nitrous gases with a concomitant drastic color change from yellow to pink and regenerated by treatment with dithionite, making it a potential sensing material for oxidant gases. Inspired by this development and the easy preparation of DHBPTz [21–24], it was hypothesized that the use of DHBPTz in a microfluidic analytical device can produce a simple new procedure for nitrite determination.

This work demonstrates a paper-based microfluidic colorimetric device for the determination of nitrite in water samples based on the dihydrotetrazine chemistry and the redox character of nitrite using a very simple operational procedure with a digital

camera or smartphone. Microfluidic analytical devices have some advantages over other analytical approaches such as providing reliable measurements with efficiency and speed using small volumes of sample solutions [37]. Microfluidic paper-based analytical devices (μ PAD) are based on the creation of hydrophilic micro-channels on cellulose-type substrates by means of two main types of techniques that form hydrophobic boundaries using materials such as wax, polymers and inks on hydrophilic paper or by forming physical boundaries by means of laser or knife cutting [38,39].

2. Materials and methods

2.1. Reagents and materials

Nitrite stock solution (80 mM) was prepared in water from sodium nitrite (CAS No, 7632-00-0, Merck, Darmstadt, Germany) dried at 105 °C. The solution was periodically standardized by titration with potassium permanganate. Solutions of lower concentration were prepared by appropriate dilution with water. This solution was stable for at least one month and was kept in a refrigerator in a stopped amber bottle. Other standards were 1000 mg L⁻¹ stock solutions of the ions: Al(III), Pb(II), Cu(II), Fe(III), Zn(II), Mg(II), Ca(II), and Na(I) (as nitrates) and chloride, fluoride, sulfate, nitrate, phosphate and hypochlorite as sodium salts, all purchased from Merck (Merck, Spain). Other chemicals were ethanol (CAS No, 64-17-5), hydrochloric acid (CAS No, 7647-01-0) and anhydrous citric acid (CAS No, 77-92-9) obtained from Panreac (Panreac Química S.A., Spain); tetrahydrofuran (CAS No, 109-99-9) from Sigma Aldrich (Sigma-Aldrich Química S.A., Spain); and acetonitrile (CAS No, 75-05-8), acetone (CAS No, 67-64-1), methanol (CAS No, 67-56-1) and ethyl acetate (CAS No, 141-78-6) supplied by Lab Scan (Lab-Scan, Spain). All reagents were of analytical-reagent-grade unless stated otherwise. All aqueous solutions were made using reverse-osmosis type quality water (Milli-RO 12 plus Milli-Q station from Millipore, conductivity 18.2 M Ω cm).

Filter paper (Ref. 1420, basis weight 80 g/m²; thickness 170 μ m; retention 14–18 μ m) from Filter-Lab, Barcelona, Spain, double-sided adhesive tape from Miarco (Valencia, Spain) and transparent sheets from Schwan-Stabilo (Heroldsberg, Germany) were used to create the μ PAD.

2.2. Synthesis of 3,6-bis(3,5-dimethyl-1H-pyrazol-1-yl)-1,4-dihydro-1,2,4,5-tetrazine (DHBPTz)

2.2.1. Triaminoguanidinemonohydrochloride [21]

Hydrazine monohydrate 6.82 g (136.4 mmol) was added to a suspension of 3.82 g (40.0 mmol) of guanidine hydrochloride in 1,4-dioxane (20 mL) at room temperature. The reaction mixture was then stirred under reflux for 2 h. The reaction mixture was cooled to room temperature, filtered, washed with 1,4-dioxane and dried. 5.0 g (85%) of white powder was obtained.

2.2.2. 3,6-Bis(3,5-dimethylpyrazol-1-yl)-1,2-dihydro-1,2,4,5-tetrazine [21]

3.04 g (21.5 mmol) of triaminoguanidinemonohydrochloride in 25 mL of water was added in drops to 4.53 g (45.3 mmol) of 2,4-pentanedione. The mixture was then stirred for 1 h, with the temperature then increased to 80 °C slowly, heating and stirring for an additional 1 h. Afterward, the solution was cooled down to room temperature slowly, filtering and washing with sufficient distilled water and dried, producing 1.76 g (30%) of pure product, mp 138–139 °C (Lit. 130–131 °C) [24]; ¹H NMR (d_6 -DMSO, 300 MHz): δ (ppm) 2.18 (s, 2CH₃), 2.41 (s, 2CH₃), 6.15 (s, 2CH), 8.82

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