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Paper-based analytical device for instrumental-free detection of thiocyanate in saliva as a biomarker of tobacco smoke exposure

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ABSTRACT

This work describes a fast and simple assay for *in situ* detection of thiocyanate, i.e., a biomarker of tobacco smoke exposure, in human saliva. The assay is based on the formation of an iron(III)-thiocyanate colored complex in a paper-based sensing platform and subsequent image analysis using a scanner as detection device. Experimental parameters influencing the color intensity of the complex were fully evaluated, including the selection of detection conditions, type of paper substrate, test zone dimensions and composition as well as the stability of the paper-based device. Under optimal conditions, the detection limit was 0.06 mM of thiocyanate, and the repeatability, expressed as relative standard deviation, was 3%. The proposed method, characterized by its simplicity, portability and low sample consumption, was applied to the detection of thiocyanate in a series of human saliva samples. Average thiocyanate levels in the ranges 0.28–0.87 mM and 0.78–4.28 mM were found for non-smokers and smokers, respectively. Recovery studies were carried out at two concentration levels, showing recovery values in the range of 96.1–103.6%.

1. Introduction

The development of simple, cheap, portable and instrumental-free analytical systems represents an exciting area of research where everyday information technology and communications devices play an important role [1]. In this vein, the possibility of developing paper-based analytical devices, recently demonstrated by Prof. Whitesides and co-workers [2–5], has received an increasing attention for a wide variety of analytical applications. Paper represents an excellent substrate material for the development of portable point-of-care assays due to the ability to immobilize chemicals in its pores, high surface-to-volume ratio, as well as low-cost, availability and disposability. Paper-based analytical systems have shown special promise as platforms for the monitoring of relevant biomarkers, clinical diagnosis and environmental monitoring in resource-limited environments. A number of excellent review articles concerning fabrication methods as well as detection systems and their analytical and bioanalytical applications have been published in the last few years, showing the current knowledge and potential of paper-based systems [6–13].

A basic requirement for the development of such paper-based systems lies in the generation of hydrophobic barriers that define the hydrophilic channels and/or test zones of the paper substrate. Several fabrication methods have been reported in the literature to define the hydrophobic barriers, wax printing being one of the most commonly used [14,15]. This fabrication method enables the generation of hydrophobic barriers in paper substrates by directly printing them with wax-based ink. A heating step is subsequently applied to allow the wax to melt through the paper, thus forming the hydrophobic barrier. The latter step could be avoided by printing a highly resolved pattern on both sides of the paper substrate using a wax printer, yet a careful alignment of patterns would be required [14]. An easy alternative to wax printing involves the use of a permanent marker in combination with metal templates with specific patterns [16]. The method requires manual drawing and therefore suffers from lower throughput but, importantly, heating steps are not required and devices could be prepared *on-site*.

Thiocyanate is an important biomarker for tobacco smoke exposure [17]. The intake of inorganic thiocyanate-containing food such as milk and cheese, as well as vegetables containing glucosinolates [18,19], contributes to the presence of this anion in human body fluids. Nevertheless, thiocyanate concentration levels in physiological fluids significantly increase on increasing exposure to hydrocyanic acid present in cigarette smoke. Thiocyanate is formed endogenously as a detoxification product of cyanide by reaction of the latter with thiosulfates in the liver [17]. Chronically high levels of thiocyanate in physiological fluids have been related to local goiter, vertigo, nasal bleeding and unconsciousness [17].

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Thiocyanate anions can be found at different concentration levels in body fluids, being significantly more concentrated in human saliva. In spite of the relative complexity of this type of sample, the fact of being non-invasive and the increased concentration levels present with respect to other physiological fluids, make saliva especially advantageous for thiocyanate detection.

Several analytical techniques have been proposed for the detection of thiocyanate in saliva, including UV–vis spectrophotometry [20–24], fluorimetry [25], atomic absorption spectrometry [26], surface enhanced Raman scattering [27], ion mobility spectrometry [18], electrochemistry [28–30], ion chromatography [31], gas chromatography [32,33] and capillary electrophoresis [34,35]. However, most of the reported methodologies are time consuming, costly, hardly portable, and involve the use of large amounts of reagents and/or solvents with the subsequent generation of significant amounts of wastes.

The development of a paper-based analytical device for salivary thiocyanate detection is described herein. The fabrication method, adapted from the one proposed by Nie et al. [16], avoids the use of special equipment and enables the easy preparation of paper-based devices on the field at significantly reduced cost. Experimental variables that influence the preparation and performance of the paper-based device were fully assessed, and the applicability of the assay was demonstrated. To the best of our knowledge, this work represents the first quantitative instrumental-free detection method for salivary thiocyanate.

2. Experimental section

2.1. Reagents and apparatus

All chemicals used were of analytical reagent grade. Ultrapure water with resistivity not less than 18.2 MΩ and TOC less than 1 ppb obtained from an Ultra Clear™ TWF EDI water purification system (Siemens, Germany) was used throughout.

A stock standard solution of thiocyanate (1 M) was prepared from KSCN (Panreac, Barcelona, Spain). Working standards were daily prepared by appropriate dilution of the stock solution with ultrapure water. The Fe(III) reagent solution (1 M) was prepared by dissolving 10.1 g of $Fe(NO_3)_3 \cdot 9H_2O$ (Merck, Darmstadt, Germany) in about 20 mL of ultrapure water, followed by the addition of 2 mL of nitric acid (Panreac), and made up to 25 mL with ultrapure water.

The following reagents were used to evaluate interfering effects: sodium acetate, potassium iodide, L-cysteine and ascorbic acid from Merck; potassium dihydrogen phosphate, sodium nitrite and D(+) glucose-1-hydrate from Panreac; sodium chloride and citric acid monohydrate from Sigma-Aldrich (St. Louis, MO, USA); sodium sulfate and sodium hydrogen carbonate from Carlo Erba (Milan, Italy); potassium chloride and magnesium chloride hexahydrate from Prolabo (Paris, France); and potassium nitrate and ammonium nitrate from Probus (Badalona, Spain).

Whatman No. 1 (180 μm, 87 g m^{-2}) and No. 3 (390 μm, 185 g m^{-2}) filter papers were purchased from Whatman (Maidstone, Kent, UK) and evaluated as the substrates for the preparation of paper-based analytical devices. A Lumocolor® permanent pen 318 (Staedtler, Nuremberg, Germany), with a line width of approximately 0.6 mm, was used to obtain the hydrophobic barriers.

A Uvikon XS spectrophotometer (Secomam, Domont, France) was used for the determination of thiocyanate in saliva samples in accordance with the reference method [36]. A Force 7 microcentrifuge Denver Instrument (Norfolk, United Kingdom) was used for centrifugation of saliva samples prior to analysis.

2.2. Preparation of paper-based analytical devices

A fabrication process previously reported in the literature [16] was adapted with slight modifications for the preparation of paper-based analytical devices. Metal templates with specific patterns were not used in this case. On the contrary, a stencil containing repeated units of specific dimensions (6 × 6 mm each) was placed under a piece of Whatman No. 1 filter paper, and the detection areas were easily plotted by using a commercially available permanent marker taking advantage of the translucence of filter paper. After allowing ink solvent to evaporate at room temperature, the required hydrophobic barriers were effectively formed by repeating this procedure on the other side of the filter paper. A credit card-sized device containing 40 separated test zones was obtained. Subsequently, 2 μL of the Fe(III) reagent solution was spotted in each detection zone and the obtained paper-based device was allowed to air-dry for 10 min at room temperature and stored at −20 °C into a zipper bag until analysis.

2.3. Procedure and data analysis

For each assay, 2 μL of standards and samples was spotted on the testing zones, and the paper-based device was digitized after allowing it to air-dry for 10 min at room temperature. An HP 4500 desktop scanner/printer was used throughout the work to digitize the paper-based analytical devices. It is worth noting that images could also be digitized by means of a portable scanner, a camera phone or a digital camera, as previously demonstrated in the literature [5]. After digitization, images were imported into ImageJ, a free image processing and analysis software [37]. Images were inverted in order to obtain increasing analytical signals at increasing thiocyanate concentrations and the mean color intensity of each relevant detection area quantified in RGB mode using the blue channel in ImageJ. The analytical responses corresponding to detection zones spotted with 2 μL of standards and samples were corrected by subtracting the mean intensity of a detection pad spotted with 2 μL of ultrapure water.

3. Results and discussion

The proposed method relies in the implementation of a well-known complexation reaction between thyocianate and iron(III) under acidic conditions in a paper substrate for the instrumental-free detection of the anion in saliva samples. Depending on the experimental conditions, a series of colored complexes with a general formula of $[Fe(SCN)_n]^{+3-n}$ (where $n = 1–6$) are formed, $[Fe(SCN)]^{2+}$ being mainly obtained in the presence of excess Fe(III). The evaluation of those experimental parameters that have an impact on the fabrication and performance of the paper-based analytical device is shown below.

3.1. Evaluation of experimental parameters

The influence of the color mode detection on the analytical signal was firstly assessed. Mean intensity values were obtained with this purpose at the appropriate test zones in both grayscale and RGB color images by using ImageJ. The scanned images were converted to grayscale images or analyzed in RGB color under the same conditions using the red, green and blue channels, respectively. The obtained results, together with typical histograms obtained using grayscale and RGB color images, are shown in Fig. 1. It can be observed that the mean color intensity for the iron(III)-thiocyanate complex increased in the order red < grayscale ~ green < blue. Therefore, the blue channel was selected for subsequent studies as it yielded the highest sensitivity