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Simple and ultrasensitive microplate method for spectrofluorimetric determination of trace resorcinol

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ABSTRACT

In this paper, a new spectrofluorimetric method is proposed for the fast, simple, and accurate determination of trace resorcinol in the microplate format (high-throughput screening). The analytical method is based on the formation of a coumarin derivative with methyl acetoacetate via a Pechmann condensation reaction. Experimental conditions have been optimized, and excellent analytical performances have been achieved with a limit of detection of 0.46 μg L^{-1}, a wide linear range between 1.5 and 1000 μg L^{-1} and a relative standard deviation of 2.01% ($n = 10$).

This method is remarkably specific for resorcinol compared to other phenols and hydroxyphenols and suffers from low interferences from other potential matrix components. The proposed protocol was finally applied and validated on real samples such as wastewater or pharmaceutical/cosmetic samples.

1. Introduction

Phenolic compounds are a class of organic contaminants that are of great concern for environmental or human health issues. Several spectroscopic methods are available for the determination of total phenolic compounds, including Prussian Blue, *o*-phenanthroline, or 4-aminoantipyrine [1,2]. Nevertheless, methods for the individual determination of these compounds can be very valuable tools to study or understand their environmental behavior, evaluate their toxic effect, or quantify a specific compound in different matrices.

Among these phenolic compounds, resorcinol (benzene-1,3-diol) has received special attention due to its toxicity and significant occurrence in the environment. Resorcinol has indeed natural sources, being a monomeric by-product of the reduction, oxidation, and microbial degradation of humic substances [3]. It is thus often found in natural waters used for tap water production, contributing to trihalomethane potential formation when these waters are chlorinated [4,5]. Apart from these natural origins, resorcinol is produced worldwide at increasing levels (from 40 000 tons in 1990 to 45 000 tons in 2000) [6] and is used in various industrial applications (rubber industry, adhesives, dyes, speciality chemicals, pharmaceuticals) [7].

Resorcinol is a proven endocrine disruptor and has been listed as a category 1 substance (substance of high concern) in the European Union priority list of substances for further evaluation of their role in endocrine disruption [8]. Toxicity for human health include dermatitis, catarrh, convulsion, or cyanopathy, and strict limitations has been set for resorcinol concentration in pharmaceutical products such as shampoos and hair lotions (0.5% limit) [9]. Serious implications of the deleterious impacts of resorcinol exposure on human health and the corresponding emergency measures to be adopted have been discussed in a document by World Health Organization (WHO) [10]. Moreover, it has been proven that resorcinol has the lowest biodegradation rates with aerobic biodegradation processes among all dihydroxybenzene isomers, resulting in increased threats for final environmental impacts [11]. Therefore, development of simple analytical methods for specific resorcinol determination has a great interest for several types of applications, such as industrial waters, wastewaters, and hair lotions.

The most common and reliable methods for the determination of resorcinol are high-performance liquid chromatography (HPLC) [12–14] and gas chromatography [15]. These methods are efficient but require expensive instruments; therefore, cheaper and simpler alternatives are highly desirable. In recent years, several methods have been described for the alternative determination of resorcinol in different samples, such as electrochemistry [16,17], spectrophotometry with nanoparticles [18] or first-derivative methods [19], direct spectrofluorimetry [20] or inhibition-based spectrofluorimetry [21,22]. Although all these methods have interesting properties for detection of resorcinol, they all suffer from relatively low sensitivity and high limit of detection of about 0.1 to 1 mg L^{-1}, a narrow linear dynamic range (less than 2 orders of magnitude) and poor or undefined specificity towards other phenols and other hydroxyphenols isomers. Thus, the development of a simple, specific, ultrasensitive, and fast method for the detection of resorcinol still remains a great challenge.

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In this work, a very simple and sensitive spectrofluorimetric method is described. The method is based on the reaction of resorcinol with a β-keto ester in acidic medium which yields a fluorescent coumarin (Pechmann condensation). Fluorescence is measured after 5 min at an excitation and emission wavelength of 332 nm and 431 nm, respectively. The simplicity of this protocol enables its implementation in microplate wells, providing high-throughput analysis with low volumes of samples, reagents, and waste. Development of the global procedure and application on real wastewater and pharmaceutical/cosmetic products are presented herein.

2. Experimental

2.1. Reagents and solutions

All chemicals were of analytical reagent grade and used without further purification. Resorcinol, methyl acetoacetate, and ethyl acetoacetate were purchased from Alfa Aesar (Schiltigheim, France), catechol, hydroquinone, phloroglucinol, phenol, 4-nitrophenol, 2-chlorophenol, and ascorbic acid were from Sigma-Aldrich (Saint-Quentin Fallavier, France), Suprapur sulfuric acid from Merck (Darmstadt, Germany). Resorcinol and other phenolic compounds stock standard solutions and working solutions were prepared in ultrapure water (Millipore, resistivity >18 MΩ cm).

2.2. Microplate instrumentation

Microplate fluorescence measurements were carried out on a microplate reader (Infinite M200, Tecan France SAS, Lyon, France) equipped with an excitation and emission double monochromator (bandwidths of 9 nm and 20 nm for excitation and emission monochromator, respectively) and controlled by i-control™ software (Tecan). Fluorescence detection was performed from above the microplate wells (top configuration), at $\lambda_{ex} = 332$ nm and $\lambda_{em} = 431$ nm. Operating temperature was set to 25 °C. Other parameters were as follows: gain: 90; number of flashes: 25; integration time: 20 μs. Fluorescence intensities were expressed in arbitrary units (a.u.). Polystyrene black 96 flat-bottom well microplates (Fisher Scientific, Illkirch, France), with a maximum capacity of 375 μL for each wells were used. In order to avoid degradation of the microplate reader due to sulfuric acid use, microplates were shaken on an external vortex mixer (Vortex Genie 2, Scientific Industries, New-York, USA), placed in the microplate reader only for fluorescent measurements and then quickly removed and stored under a fume hood.

2.3. Optimized protocol for resorcinol determination in aqueous samples

Seventy-five microliters of sample or resorcinol standard solution was dispensed into the wells of the microplate, followed by 75 μL of sulfuric acid and 10 μL of methyl acetoacetate. The plate was shaken for 5 min at room temperature on the vortex mixer, and fluorescence was subsequently measured at $\lambda_{ex} = 332$ nm and $\lambda_{em} = 431$ nm in the microplate reader. Resorcinol concentrations were determined using the linear calibration curve obtained with standards. All experiments were performed in duplicate.

3. Results and discussion

3.1. Formation of the fluorescent product

Pechmann condensation is a well-known organic reaction used for synthesis of various coumarins. Reaction scheme with resorcinol is depicted on Fig. 1, with formation of 7-hydroxy-4-methylcoumarin (4-methylumbelliferone). Application of this reaction for analytical purposes appeared very interesting to us since the formation of highly fluorescent products from non- or poorly fluorescent analytes/reagents is a method of choice for attaining high sensitivity.

The selection of optimum excitation and emission wavelengths was carried out on the fluorescent product formed under the reaction conditions. 7-Hydroxy-4-methylcoumarin is a well-known fluorophore with reported optimum excitation and emission wavelengths of 340–365 nm and 430–450 nm, respectively [23]. For optimum fluorescent measurements in black microplates, one has to investigate not only the maximum of intensity for a given standard, but also the intensity of a blank sample (with no standard, which takes also into account the autofluorescence of the microplate material). Fig. 2 displays the best set of wavelengths by measuring the optimum signal/blank ratio, with final wavelengths set at 332 nm and 431 nm for excitation and emission, respectively.

3.2. Optimization of experimental conditions

3.2.1. Sulfuric acid concentration

Classical conditions for coumarin formation via Pechmann condensation require sulfuric acid to reach good yields in a reasonable time. Many alternatives have been developed to try to avoid the use of strong acid. Catalysts such as $ZrOCl_2.8H_2O/SiO_2$ [24], zeolite [25] or boron trifluoride [26] have been used in organic solvents or in solvent-free procedures, but they are clearly unsuitable for our analytical purpose in aqueous medium (aqueous samples containing resorcinol). One catalyzed procedure in water has been described with acidic ionic liquids [27], but it requires heating at 80–100 °C for 1 h to reach coumarin formation. These reaction conditions are also clearly inapplicable with plastic microplates, and reaction times are too long for fast analyses. Considering drawbacks of these alternatives, we chose to keep sulfuric acid to promote fast coumarin formation. A waste disposal protocol has to be set up for sulfuric acid, but it is a routine procedure for an analytical laboratory and potentially less constraining than toxic elements as catalysts. Moreover, low volumes used due to microplate format enable strong reduction of acidic waste generated.

Various volumes of sulfuric acid were tested (with a fixed volume of sample) in order to optimize the signal/blank ratio on a resorcinol standard solution (Fig. 3A). Best results were obtained for H_2SO_4/resorcinol volume ratio close to 1. Lower ratios resulted in incomplete coumarin formation, while higher ratios implied higher blank values due to residual fluorescence in the sulfuric acid, with no change in the resorcinol standard response.

3.2.2. Methyl acetoacetate concentration

Methyl acetoacetate is added directly without any dilution, and influence of the addition of increasing volumes on the fluorescence

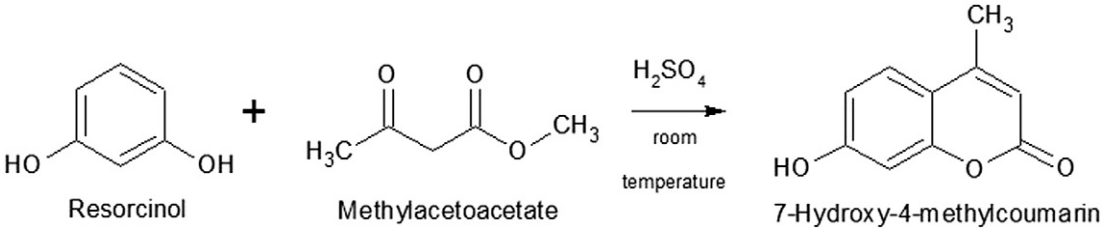

Fig. 1. Schematic depiction of the formation of a fluorescent coumarin with resorcinol.