

MESTRADO CONTROLO DE QUALIDADE

**Synthesis of fluorescent carbon nanoparticles: optimization based on quantum yield and preliminary studies for future application in the chemical control of nitrites**

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The control of food quality is an urgent need that is related to ensuring human health, helping to prevent diseases and unwanted effects, looking for safe food consumption. In the industrial food sector, food additives from different categories are frequently used, such as food preservatives, which include nitrite salts. These substances must be present in compliant categories and standardized by regulatory bodies and these levels need to be checked frequently, justifying the necessity of analytical techniques to detect nitrite ions compounds in varied food matrices, as water, vegetables, and meats.

Given this context of nitrite ions' detection possibilities, carbon dots have emerged as a new versatile nanomaterial, with highly attractive luminescent properties applicable in different chemical and biochemical areas, being a possible promising approach for chemical sensing including ensure food safety.

In this investigation work, the possibility of using the fluorescent properties of carbon dots for the determination of nitrite ions by fluorescence quenching generated by the reaction with nitrite ions was studied. A simple and easy route to synthesize carbon dots was proposed, through a microwave-assisted green synthesis method, aiming at the maximization of the quantum yield and monitoring of the fluorescence emission and absorption spectra. Also, some preliminary tests involving the reaction between the as-synthesized carbon dots with nitrites and evaluation of some experimental conditions was performed.

The optimization of the parameters time of reaction, temperature, microwave power and molar ratio of reagents, allowed to synthesize carbon dots with a relative quantum yield of approximately 70%, and a fluorescence emission wavelength at 440 nm. The preliminary assays between carbon dots and nitrite ions confirmed that by using a concentration of 0.02 mg/mL of carbon dots and a reaction time of 25 minutes, the detection of 1 ppm of nitrites was attainable, by monitoring the fluorescence quenching of the carbon dots.

**Keywords:** carbon dots; optimization; synthesis; fluorescence quenching; nitrites; food safety.

O controle de qualidade alimentar é uma necessidade urgente que se relaciona com a garantia da saúde humana, auxiliando na prevenção de doenças e efeitos indesejados, de forma a visar um consumo alimentar seguro. No setor alimentar industrial são frequentemente utilizados aditivos alimentares de diversas categorias, como os conservantes alimentares, onde se enquadram os sais de nitritos. Essas substâncias devem estar presentes em categorias adequadas e padronizadas por entidades reguladoras, sendo necessário que esses níveis sejam frequentemente verificados, recorrendo-se então a técnicas analíticas para detecção de iões nitrito nas respetivas matrizes alimentares, como água, vegetais e carnes.

Dado esse contexto de possíveis vias de detecção de iões nitrito, pontos de carbono surgem como novos nano-materiais versáteis, com propriedades luminescentes altamente atrativas, aplicáveis em diferentes áreas químicas e bioquímicas, sendo uma abordagem promissora possível para sensoriamento químico a fim de garantir a segurança alimentar.

Neste trabalho de investigação foi estudada a possibilidade de utilização das propriedades fluorescentes dos pontos de carbono para determinação de nitritos pela extinção de fluorescência gerada pela reação com iões nitritos. Foi proposta uma rota simples e fácil de obter pontos de carbono, através de um método de síntese verde assistido por micro-ondas, objetivando a maximização do rendimento quântico de fluorescência, monitorando os espectros de absorção e emissão de fluorescência. Além disso, testes preliminares envolvendo reações com nitritos e os pontos de carbono sintetizados foram realizados, com avaliação de algumas condições experimentais.

A otimização dos parâmetros tempo de reação, temperatura, potência do micro-ondas e rácio molar entre os reagentes permitiu pontos de carbono sintetizados com um rendimento quântico relativo de aproximadamente 70% e um comprimento de onda de emissão máximo de 440 nm. Os ensaios iniciais permitiram concluir que com uma concentração de 0,02 mg/mL de pontos de carbono e um tempo de reação de 25 minutos, a detecção de 1 ppm de iões nitritos é atingível, sendo verificada pela extinção da fluorescência dos pontos de carbono.

Palavras-chave: pontos de carbono; otimização; síntese; extinção de fluorescência; nitritos; segurança alimentar.



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## List of abbreviations

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CDs	Carbon dots
CNDs	Carbon nanodots
CQDs	Carbon quantum dots
DET	Dexter energy transfer
EFSA	European Food Safety Authority
FL	Fluorescence
FRET	Förster resonance energy transfer
FTIR	Fourier Transform Infrared Spectroscopy
GQDS	Graphene quantum dots
IARC	International Agency for Research on Cancer
IFE	Inner filter effect
LOD	Limit of detection
PDs	Polymer dots
PET	Photoinduced electron transfer
QY	Quantum yield
SET	Surface energy transfer
TEM	Transmission electron microscopy
WHO	World Health Organization
USDA	United States Department of Agriculture
USEPA	United States Environmental Protection Agency
UV	Ultraviolet
UV-Vis	Ultraviolet-visible

# **THEORETICAL PART**

# 1. Introduction

---

In this chapter, the context in which this work was developed is broadly addressed: the existence of nitrites and the respective human contact with them, explaining the importance of their detection, as well as the different possibilities for analytical determination. Then the insertion of carbon dots (CDs) as auxiliaries in new fluorescent sensing methods and the particularities of their syntheses and physical-chemical characteristics.

## 1.1. Nitrates and nitrites

Nitrate and nitrite ions are naturally present in the environment and in food but can also be intentionally used in industrial and agricultural sectors in the form of nitrate and nitrite salts. Consequently, they act on human physiological systems, through consumption and contact with them (1).

Processed foods require several constituents so that their final quality can be guaranteed, and their ingesting considered safe. The use of nitrate and nitrite ions in the food sector is related to their preservative properties, since they are classified as food additives capable of preserving food, prolonging its shelf life. They inhibit the growth of unwanted microorganisms, such as *Clostridium botulinum*, bacteria responsible for triggering toxins that cause botulism. So, nitrite salts (sodium nitrite and potassium nitrite) are repeatedly used as food additives (2) and are both allowed by European Union according Commission Regulation 1129/2011 (3).

In addition to conservation effects, nitrite ions can also improve color, texture and flavor aspects of meat products (4). When consumed in adequate amounts, nitrites can be considered beneficial to the body, as they can act in the regulation of blood flow, for example (5), a fact associated with the production of nitric oxide (2). The huge problem is when these amounts exceed the appropriate limits for human consumption, being of extreme importance for the health and safety of the human being the frequent chemical monitoring for the presence of such ions in food matrices, to ensure that they follow current regulations (6). Only the nitrate ion is not identified as toxic, but through the actions of bacteria, mainly in saliva or the gastrointestinal tract, it can be converted to nitrite (2).

This chemical control is even more important considering that, in an acidic environment, nitrite ions give rise to nitrous acid, which, through reactions with secondary or tertiary amines, can originate carcinogenic compounds, namely nitrosamines, that are harmful to individuals as expounded in the reaction in figure 1 (7).

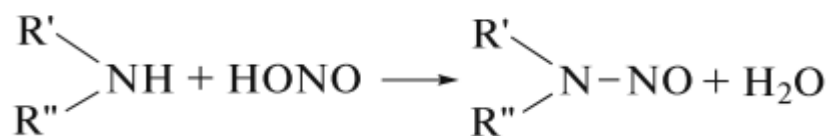


Figure 1: Reaction of amine and nitrous acid with formation of nitrosamines. Reprinted from (7), with permission from Springer.

According to the International Agency for Research on Cancer (IARC), nitrite ions are placed in group 2A, classified as a group of probable carcinogens to humans (5, 8). With its wide presence in meat foods due to the preservative and inhibiting action of undesirable bacteria, it is crucial that its levels present in these foods are analysed by efficient analytical methods so that food consumption can be considered benign (9).

In addition to the damage associated with cancer from nitrosamines, a temporary blood disorder called methemoglobinemia can also occur, translated by the difficulty in transporting oxygen from the blood, associated with the presence of methaemoglobin and reduced levels of haemoglobin (10, 11). This disease is even more worrying in babies, given the impaired neural functioning due to insufficient oxygenation, which can be fatal (blue baby syndrome) (11). The nitrite ion is, therefore, often classified as a toxic component widely present in environmental, food and biological environments, capable of causing immense harm to human health (12).

To evaluate such presence of nitrites, is necessary to compare the results of chemical analyses with the values established by the regulatory authorities of food safety, environment, and human health. These references are also important to know if the new developed methods reach the required sensitivity to identify the toxic levels of the referred ion.

The European Food Safety Authority (EFSA) sets the maximum level of nitrite ions in meat products at 150 mg/kg (13). The Scientific Committee of the European Union establishes a limit of 0.06 mg/kg for daily consumption of nitrite ions ( $\text{NO}_2^-$ ). The United States Environmental Protection Agency (USEPA) claims that the maximum amount of  $\text{NO}_2^-$  allowed in fresh water is 1 mg/L, while the World Health Organization (WHO) sets 3 mg/L (14, 15). The United States Department of Agriculture (USDA) also sets  $\text{NO}_2^-$  limits in some food products, such as: 120 ppm for bacon, 220 ppm for ham and 650 ppm for dry-cured meat products (16). Nitrites can be used in dairy products from exogenous fonts, and in this case, the limit permitted by Commission Regulation 1129/2011 for cheese, for example, is 150 mg/kg (3).

### 1.1.1. Methods for nitrite ion chemical control

Several methods are known to detect and quantify nitrites in different matrices, which can be food, environmental or biological. Even limited to food matrices, spectrophotometric (17, 18), chemiluminescent (19, 20), electrochemical (21, 22), chromatographic (23), capillary electrophoresis methods (24, 25), among others, are described in the scientific literature.

However, for reasons that may involve high equipment cost, long treatment processes, difficulties in practical applications and low limits of detection (LOD), the existing methods to detect nitrites can be improved and methods involving nanotechnology emerge as a possibly more advantageous and certainly innovative option (26) with the creation of specific optical and electrochemical sensors (27).

From nanotechnology, the creation of structures on a nanometric scale is possible, which result in nanomaterials with physical and chemical characteristics different from the source material. Examples are noble metal nanoclusters, semiconductor quantum dots and carbon dots (28, 29).

### 1.2. Carbon dots

Carbon dots are defined as zero-dimensional carbon-based nanomaterials, with a size less than 10 nm, with luminescent properties, abundant external morphology and ample possibilities for synthesis and sources of raw materials (30). Regarding the names used for carbon-based nanomaterials, it is possible to find specific subdivisions such as: graphene quantum dots (GQDS), carbon nanodots (CNDs), polymer dots (PDs) and carbon quantum dots (CQDs) (31). Despite all these different nanomaterials are carbon-based, they differ in their structural 3D arrangement of the atoms of carbon, and in the type of chemical bonds between the atoms, giving rise to nanomaterials of some distinct physical-chemical properties.

Chunlei Xia et al (31, 32), in a review published in 2019, included the designation of carbonized polymer dots (CPDs), differentiating them by having a hybrid body structure of polymer and carbon instead of the main structure only of carbon, obtained through the incomplete carbonization of the polymer agglomerates. The different possible structures related to CDs depend on the elements incorporated, the surface state and the form of heating, which influence the level of carbonization (33).

The CDs are spherical or nearly spherical structures and sizes differ due to factors related with the syntheses processes such as reaction time and temperature, for example. They may have carbonyl, amine, and hydroxyl groups in their structure, which are responsible for the



hydrophilic character associated with CDs, making them convenient for composition with other materials without phase separation (31, 32).

The elements carbon, oxygen, nitrogen and hydrogen are the main constituents of the structure of CDs, which confer low toxicity and biocompatibility (34), making those carbon materials more attractive compared to traditional metallic quantum dots such as lead or cadmium (31, 35).

CDs can exhibit excitation-dependent fluorescence (FL) emission (36) (Figure 2), although the luminescent origin of these nanomaterials is not yet evident (37). There are different possibilities to explain the origin of luminescence characteristic of CDs, with the most explored proposals being: quantum confinement effect, surface defects, molecule-like states, synergistic effects of luminescence centres. There are reports stating that as the size of the CDs decreases, the interval between the energy states of the carbon nucleus increases, which causes a shift in the CDs' emission wavelength (35, 38).

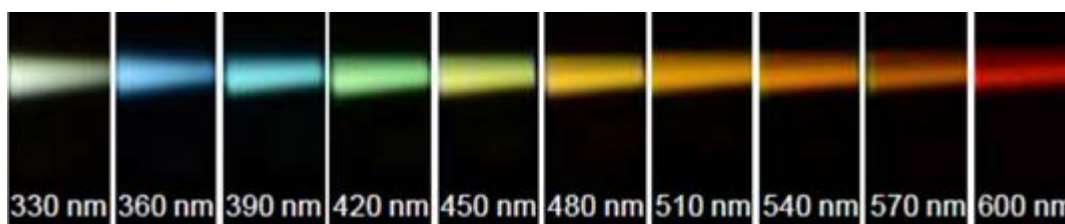


Figure 2: Possible fluorescence of CDs with excitation at different wavelengths. Reprinted from (39), with permission from Springer.

The light emitted by CDs after absorption of electromagnetic radiation in the ultraviolet (UV) zone is mostly blue, that is, they are mostly fluorescent in the region of shorter wavelengths of the light spectrum (40). The varied wavelength possibilities emitted by CDs provide advantages according to the objectives. As an example, CDs that emit red light can provide adequate fluorescence for in vivo imaging using visible light as an excitation source, unlike CDs that emit blue light. In CDs with blue light emission, the excitation source would have to come from a region of the light spectrum with a shorter wavelength (as in the UV region), which may be harmful to live specimens. Another issue is the biological sample having a weaker autofluorescence in the red region of the light spectrum, resulting in a limited image (33).

The fluorescence property displayed by CDs added to their low toxicity makes this new class of nanomaterials attractive for application in different fields, such as bioimaging and chemical and microbial detection, for example (35). In the case of the food sector, where there is a particular interest in ensuring safety through the control of food quality, CDs have a great potential, allowing detection of contaminants, additives, pesticides, toxins, etc., with high sensitivity and selectivity (37).

An interesting review available in 2019, produced by Xingbo Shi et al (41), demonstrated the great applicability of CDs as allied sensors in food safety, demonstrating their use for detection of various metals ( $\text{Fe}^{+3}$ ,  $\text{Cu}^{+2}$ ,  $\text{Ag}^+$ , etc.), as well as bacteria, pesticides and other compounds of interest (41). When it comes to the detection of nitrites, there are still few studies developed from the use of carbon dots (42).

### 1.2.1. Carbon dots synthesis methods

There are two main CDs synthesis mechanisms: top-down and bottom-up and the carbon dots formation through each of these methods is demonstrated in figure 3. The technique called top-down consists of a more physical process, whose carbon source is a bulk material (bulk), which is reduced to the nanometric scale. Laser ablation and electrochemical exfoliation are examples of this method. Graphite materials are typically used as the carbon sources in this synthetic technique (43). The technique classified as bottom-up, on the other hand, is related to a more properly chemical process, where the starting point is small molecular compounds, with carbonization, pyrolysis, and hydrothermal synthesis being examples of this type of method (44, 45).

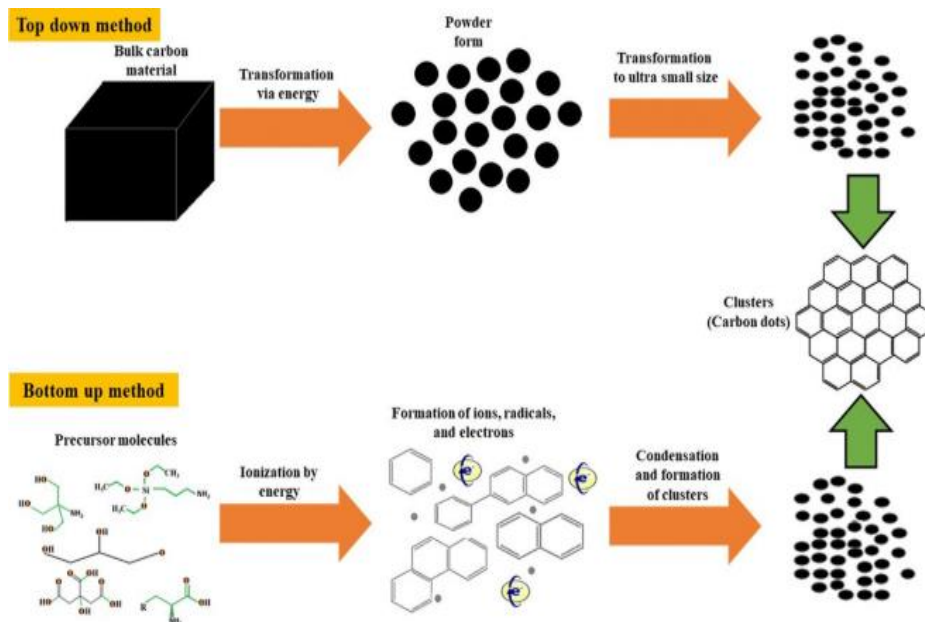


Figure 3: Top-down and bottom-up mechanisms for carbon dot synthesis. Reprinted with permission from (46), published by Catalysts, MDPI.

When compared, top-down CDs perform better, but the procedures involved are more complex and require more costly instrumentation. The bottom-up has been the most used technique in recent years, seen as a technique that allows for large-scale synthesis (32).

Additionally, the sizes of CDs can be easily manipulated through the control of the synthesis conditions in thermal or pyrolytic treatments. There is also a greater source of carbon available, at a low cost and biocompatible, such as glucose, amino acid, citric acid (CA), ascorbic acid, citrate, chitosan, proteins, fruit juices, food peels, etc (47-49). Other advantages presented by the bottom-up method are the reduced time of synthesis, being more ecologically correct and the possibility of modifying the surface and composition of the CDs (33). Figure 4 presents a summary of some methods classified as bottom-up and top-down and their respective advantages and disadvantages.

		Method	Advantages	Disadvantages
Approaches for the synthesis of CQDs	Top - Down	Arc discharge	Attainable method	Harsh conditions, low quantum yield
		Laser ablation	Effective & effortless method	Large raw material, poor control & low yield
		Plasma treatment	Attainable method	Harsh conditions, poor control
		Electro-chemical	Stable method	Complex method
	Bottom - up	Pyrolysis	Simple, large substrate scope	Harsh condition
		Microwave	Convenient & eco-friendly method	Poor control over size
		Sono-chemical	Sustainable, eco-friendly	Poor control, less efficient
		Hydrothermal /Solvothermal	Easy, cheap, non-toxic method	Poor control over size & low yield

Figure 4: Methods, advantages and disadvantages of carbon dot synthesis. Reprinted with permission from (50). Copyright 2021 American Chemical Society.

The CDs found in the scientific literature between 2017 and 2021 for nitrite detection were mostly synthesized by hydrothermal/solvothermal treatment and microwave assisted method. A brief introduction to these methods is discussed following.

#### 1.2.1.1. Hydrothermal/solvothermal CDs synthesis method

Hydrothermal/solvothermal treatments are recurrently used, considered simple, possibly ecological (when using renewable resources, for example, such as aloe, fruit peels, etc.) (41), and of low cost (27). The hydrothermal method is based on the reaction at elevated temperatures (between 100 and 200 °C), usually in a Teflon-lined autoclave without the presence of air, with organic precursors and an appropriate solvent, where the carbonization process takes place (28, 51).

Among the synthetic methodologies classified as bottom up, the hydrothermal treatment is the carbonization process preferentially chosen for its versatility, low time required, with good cost-benefit through easily accessible carbon precursors (29). In the solvothermal method, an organic solvent is used and kept under high temperatures and pressures, which allows the interaction with the precursors, but it can also be considered an approach limited by high temperatures and toxic by the solvents used (41).

#### 1.2.1.2. Microwave-assisted CDs synthesis method

An alternative, fast and localized heating method is through microwave radiation, that is an electromagnetic radiation capable of exciting the molecules, with an easier interaction with carbon-based compounds (51). The materials used, unlike hydrothermal carbonization, are non-metallic, which enhance the digestion of organic materials (33). The use of microwaves as an aid to the CDs synthesis process is a faster, simpler, environmentally correct, and economical method due to energy savings (48), with the associated time savings being a distinctively attractive factor (41).

#### 1.2.2. CDs characterization

Different methods can be used to characterize the carbon dots, such as Fourier transform infrared spectrophotometry (FTIR) to identify the functional groups present, transmission electron microscopy (TEM) for information about the morphology and size of nanostructures at the nanoscale, and ultraviolet-visible (UV-Vis) and fluorescence spectrophotometry for optical behavior analysis (30).

### 1.2.2.1. Optical properties

The CDs have optical absorption in the UV region in the range of 260 to 320 nm, extending to the visible range. In the wavelength range of 230 to 280 nm, the  $\pi-\pi^*$  transitions of the aromatic C=C bonds are associated and in the range of 300 to 370 nm the  $n-\pi^*$  transitions of the C=O bonds (52). When passivated, the corresponding range is increased accordingly. In general, despite the different structures resulting from the synthesis conditions, CDs have high photostability and a continuous fluorescence emission with a long duration, but which can be easily affected by pH (48).

Regarding fluorescence, it can be considered that the absorption energy is superior to the emission energy since the emission wavelength is higher than the absorption wavelength (52).

### 1.2.2.2. Quantum fluorescence yield

The quantum yield (QY) is an important parameter for the characterization of CDs, where it is desired that these values be high. From high QY values there is a better location of particles for a higher resolution, as there is an increase in the signal-to-noise ratio when more photons are being emitted per particle (33).

The QY value can be obtained relative to a naturally luminescent reference substance or absolutely. The absolute value measurement does not need a reference material, measurements are made of the actual number of photons absorbed and emitted by the compound under study, through an integrating sphere (33).

The equation (1) demonstrates the calculation of the absolute fluorescence yield ( $QY_{abs}$ ), where  $L_{emission}$  refers to the number of photons emitted from the fluorescent particle and  $E_{sample}$  and  $E_{solvent}$  the number of light photons used to excite the fluorescent particle and the solvent, respectively (53).

$$QY_{abs} = \frac{\int L_{emission}}{\int E_{solvent} - \int E_{sample}} \quad \text{Equation (1)}$$

### 1.2.2.3. Doping with heteroatoms

It is possible to improve the performance of the optical and electronic properties of CDs by performing a doping with precursors containing heteroatoms, which when introduced into the carbon points, change the skeleton and chemical structure of the surface matrix, enhancing the characteristics of the nano compounds in question (31). This doping can provide more active sites like -OH, -COOH, and -NH<sub>2</sub> on CDs surfaces (54).

As examples, doping with N, S, P and B is feasible, and doping with N (can be through urea, acetonitrile, ethylenediamine (EDA), pyridine, etc) is detachable and widely used, as it has the advantages: comparable size between N and C, the fact that nitrogen has five electrons in the valence layer for binding with carbon atoms and increasing the quantum yield of fluorescence (46, 55). Doping with N is also attractive due to its abundant natural presence in bio-derived resources or through low-cost and low-complexity synthetic routes (50). In 2017, Wan Zhou et al (31) published a study in which CDs were doped with S and N through a hydrothermal method from the reaction of phenylenediamine and sulfuric acid.

### 1.3. Nitrites and CDs

Several studies involving the determination of nitrites by reaction with CDs and monitoring the variation of fluorescence, are reported. The table 1 brings together temperature, time and pH conditions used by the authors to synthesize carbon dots with each method used, reagents, and quantum yield obtained, along with the calculated detection limits and linear detection range to determine nitrites, in most recent studies (2017-2021) published in the scientific literature in food matrices.

Table 1: Parameters obtained and conditions for the synthesis of carbon nanoparticles for determination of nitrites in food samples.

Reference	Method	Reagents	QY (%)	Detection limit	Detection range	Temperature (°C)	Time (min)	pH
(34)	hydrothermal synthesis	tris-(hydroxymethyl)-aminomethane and urea	55	13.5 $\mu\text{mol/L}$	15–1110 $\mu\text{mol/L}$	210	180	5.0-10.0
(9)	one-pot hydrothermal route	2,3-diaminobenzoic acid hydrochloride and sulfuric acid 98%	8 and 18	31.61 $\text{nmol/L}$	0.1–100 $\mu\text{mol/L}$	200	120	2.0
(35)	solvothermal method	2,3-diamino pyridine and dimethylformamide solution	21	2.8 $\text{nmol/L}$	0.005–0.04 $\text{mmol/L}$	180	600	3.0-9.0
(15)	one-step hydrothermal method	ascorbic acid and benzylamine	12.4	43 $\text{nmol/L}$ (800 nm) and 0.65 $\mu\text{M}$ (375nm)	0–14 $\mu\text{mol/L}$	180	300	1.6
(36)	one-pot hydrothermal method	anhydrous sodium sulfate, citric acid, and sodium phytate	15.69	0.3 $\mu\text{mol/L}$	0.7–9 $\mu\text{mol/L}$	180	420	6.0
(37)	acid-assisted heating method (polymerization)	ascorbic acid, phosphate acid and polyethylene imine	3.8	0.55 $\mu\text{mol/L}$	2–100 $\mu\text{mol/L}$	90	1200	1.83-11.81

Reference	Method	Reagents	QY (%)	Detection limit	Detection range	Temperature (°C)	Time (min)	pH
(38)	microwave-assisted Carbonization	citric acid and triethylenetetramine	39.5	0.025 µmol/L	0.1–75 µmol/L	-	50	2.0
(40)	one-step hydrothermal	acriflavine	36.13	1.6 nmol/L	50 nmol/L–10 µmol/L	180	720	Acidic conditions
(42)	reaction with microwave	ethylenediamine and citric acid	-	6.5 µg/L	20–500 µg/L	-	4	1.5
(56)	hydrothermal treatment	EDA and citric acid	-	40 nmol/L	0.2–20 µmol/L	180	300	2.0
(57)	one-step hydrothermal carbonization method	m-phenylenediamine and ethanol	31.58	0.018 µmol/L	0.063–2.0 µmol/L	180	720	Acidic conditions
(58)	hydrothermal carbonization	p-phenylenediamine and CA	-	19.3 nmol/L	0.02–40 mmol/L	210	660	2.5
(59)	one-step hydrothermal method	3-aminophenol, ethanol, and hydrochloric acid	2.5	0.23 µg/mL	0.4–20 µg/mL	140	720	3.0
(60)	one-step hydrothermal treatment	p-phenylenediamine and citric acid	4.8	0.65 µmol/L	8–100 µmol/L	180	240	3.0–12.0



Regarding the use of carbon dots for detection of nitrites, optical sensors, or electrochemical sensors were developed. Sensors centred on CDs can be based on quenching or preventing the extinction of the fluorescence emission from CDs in contact with the analyte in question, in this case, the nitrite ion. The consequent quenching is then associated with a charge transfer process (61). That is, the interactions between the analyte and the carbon dots are responsible for decreasing or increasing the fluorescence and based on these principles, different mechanisms can be associated, such as: static quenching, dynamic quenching, Förster resonance energy transfer (FRET), photoinduced electron transfer (PET), Dexter energy transfer (DET), Surface energy transfer (SET) and inner filter effect (IFE) (62).

Taking advantage of the luminescent properties of CDs and framing food quality control, in 2018, Katerina K. Karali et al (34) synthesized nitrogen-doped CNDs through urea and tris-(hydroxymethyl)-aminomethane and subsequently nitrite sensing was carried out in enriched food samples, such as cheese, flour and sausages, within a linear detection range of 15 to 1110  $\mu\text{mol/L}$  and detection limit of 13.5  $\mu\text{mol/L}$ . The CNDs had a quantum yield of 55% and the method was considered environmentally sustainable. In 2019, researchers Juanjuan Liu et al (9) developed a CD-based ratiometric probe to control nitrites in food samples, testing samples of bacon, sausage, pickles, and milk. The determination allowed a detection limit of 31.61  $\mu\text{mol/L}$ , with a concentration range with linear response ranging from 0.1 to 100  $\mu\text{mol/L}$ . The CDs were hydrothermally synthesized in a single step and the detection principle was also the fluorescence quenching, based on the static quenching effect.

In the study produced in the year of 2020 by Minghui Zan et al (35), the solvothermal method was used with 2,3-diamino pyridine and dimethylformamide to produce green-fluorescent carbon dots, which were used in water, urine, and serum samples to determine the presence of the nitrite ion by the fluorescence quenching mechanism. The CDs were selected because of their optical properties, such as high photostability and luminescent emission, suitable for the development of a probe. The fluorescence extinction can be observed as the nitrite concentration increases, ensuring the viability of the detection and the detection limit in aqueous medium was 2.8  $\text{nmol/L}$ .

Through an investigation published in 2017, Zhibiao Feng et al (56) hydrothermally prepared N-doped CQDs from citric acid and ethylenediamine for application as a fluorescent probe for detection of nitrites. They reported that by adding nitrite ions, the fluorescence intensity was significantly reduced, and the fluorescence mechanism was associated with the appearance of nitrous compounds under acidic conditions, the static fluorescence quenching mechanism being likely. The linear detection range for nitrite

concentration using the probe produced was 0.2 to 20  $\mu\text{mol/L}$  and detection limit of 40  $\mu\text{mol/L}$  in tap water samples.

In 2019, researchers Bao-Lin Li et al (42) produced CDs by ethylenediamine and citric acid, with the aid of microwaves, to determine nitrites in food samples (sausages). The principle of this  $\text{NO}_2^-$  determination was the extinction of fluorescence, made possible by the formation of the nitrosonium cation under acidic conditions, given the existence of primary amines on the surface of the produced CDs. The method enabled the detection of nitrite concentration in a linear range from 20 to 500  $\mu\text{g/L}$ , while the detection limit was 6.5  $\mu\text{g/L}$ .

In addition to the obvious interest in applications involving fluorescence, CDs can also be used in electrode modification for electrochemical detection of nitrite ions, as revealed by Kunxia Li et al (63) in 2019. The mentioned study was based on plant seeds as precursors for water-soluble CD preparations. The CDs were synthesized by the direct calcination method and doped with nitrogen to develop an electrochemical sensor to identify the nitrite ion, which was later tested on sausage samples. The detection limit found was low (0.23  $\mu\text{mol/L}$ ), with the CDs showing good temporal stability in relation to their response to nitrite ions. The authors concluded that the analysis of the presence of nitrites in real samples was quick and satisfactory.

#### 1.4. Purpose of experimental work

The present work was developed in the Laboratory of Applied Chemistry at the Department of Chemical Sciences of the Faculty of Pharmacy of the University of Porto, and the main objective was to synthesize CDs through a microwave-assisted single-step reaction and evaluate its potential use in the detection of nitrites in sample foods. The developed work included:

- Optimization of the ideal conditions for the synthesis of CDs aiming at the highest QY.
- Spectral characterization of the obtained CDs.
- Verification of the feasibility of detection of nitrites by fluorescence quenching by reaction with CDs.
- Preliminary studies of nitrite detection conditions.

# **EXPERIMENTAL PART**

## 2. Materials and methods

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In this chapter, the respective reagents, standard solutions, and equipment that were used in all steps of the experimental procedures are described.

### 2.1. Reagents and solutions

To prepare the solutions used in this work, purified water from a Milli-Q system (conductivity  $\leq 0.1 \mu\text{S cm}^{-1}$ ) and high analytical grade reagents without any additional treatment or purification process were used. The reagents citric acid monohydrate and quinine sulphate dihydrate were purchased from VWR Chemicals (Leuven, Belgium), ethylenediamine was obtained by Alfa Aesar (Kandel, Germany), the sulfuric acid was obtained by Fisher Chemical Scientific (Loughborough, UK) and the sodium nitrite from Sigma-Aldrich.

The standard solutions used in this work were prepared by stock dilution with micropipettes (P20, P100, P1000 and P5000 with maximum volumes of 20, 100, 1000 and 5000  $\mu\text{L}$ ) and volumetric flasks of different capacities.

### 2.2. Equipment

A pressurized microwave synthesizer (CEM<sup>®</sup> Discover SP, 2.5 GHz MW, 300 W, Matthews, North Carolina, USA) was used to perform the CDs syntheses. The synthesized CDs were characterized by ultraviolet-visible spectroscopy (UV-Vis) using a Jasco V-660 spectrophotometer (Easton, MD, USA), at wavelengths between 300 and 550 nm, and by using a quartz cell with a 1 cm optical path. For fluorescence recording a Jasco FP-6500 fluorescence spectrophotometer (Easton, MD, USA) was used and the excitation and emission slit widths were 5 nm. The samples were lyophilized in a freeze fryer Lyo Quest (Telstar, Terrassa, Spain). Both fluorescence spectrophotometer and fluorescence UV-Vis spectrophotometer were also used to calculate the relative quantum yield. The sensitivity of fluorescence spectrophotometer was adjusted according to the tests.

### 3. Results and discussions

In this chapter, the results obtained in the work are discussed, with relevant comments related to the synthesis of carbon dots, the optimization of established conditions based on the highest quantum yield, the optical characterization of CDs and the preliminary tests for the detection of nitrites.

#### 3.1. Synthesis of CDs

The CDs were obtained via a single-step synthesis with the assistance of a microwave (power fixed at 60 W) from an aqueous solution of citric acid (carbon source), ethylenediamine (nitrogen source) and water. Briefly, a mass of 8.4050 g of citric acid was weighed and dissolved in a 50.00 mL volumetric flask with water, to make the citric acid stock solution used. Ethylenediamine was used directly in a 1:1 molar ratio to citric acid and water was used to complete the 7 mL volume. The reagents were transferred in the order mentioned to a 10 mL reaction vessel, with the addition of a magnetic stirrer and reacted for 10 minutes under 150 °C. The as-prepared suspensions of CDs were frozen at -83 °C, lyophilized and the solid obtained was easily redispersed in ultra-pure water for further characterization studies and preliminary assays with nitrite ions. In figure 5 there is a representative scheme of the defined process for obtaining the CDs.

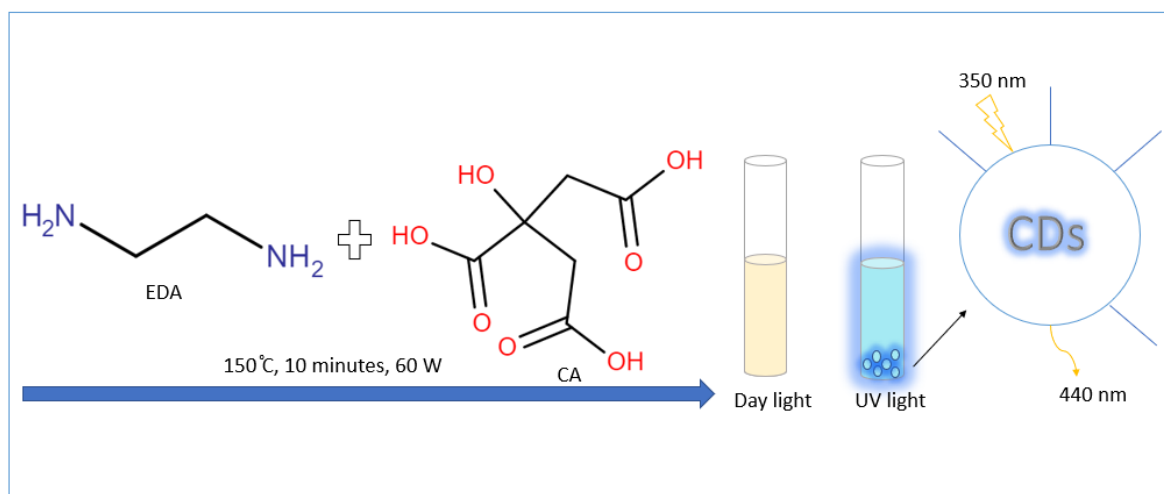


Figure 5: Scheme of synthesis of CDs for nitrite detection.

After synthesis, optical characterization of the CDs was executed, where the UV-Vis spectra showed that the maximum absorbance wavelength was at approximately 350 nm, as indicated in figure 6-a. This result was in accordance with the literature which informs

that carbon dots synthesized from ethylenediamine and citric acid absorb light principally at wavelengths under 400 nm, with the two prominent peaks at approximately 240 and 350 nm. Similar results characteristic of UV-Vis were described by Wu, X. et al (64) in 2019, where N-CQDs were obtained using the same precursors through a hydrothermal treatment and also by Liu, Wenfang et al (65), in 2020, who prepared N-CQDs by different nitrogen sources, including ethylenediamine.

In the fluorescence analysis, different excitation wavelengths (345, 348, 350, 355, 370 and 380 nm) were tested, and the fluorescence activity demonstrated being dependent on the excitation (Figure 6-b). A work developed by Lanlan Gan et al (66), published in 2020, synthesized CDs hydrothermally to determine nitrites via folic acid and nicotinic acid. During the investigation, they analysed different excitation wavelengths, between 320 and 380 nm and the CDs were also excitation-dependent, having a variation in the fluorescence intensity obtained. The same can be seen in the 2019 study conducted by Yushan Liu et al (58), in which citric acid and p-phenylenediamine were the precursors for obtaining fluorescent CDs through a hydrothermal route. When analysed the dependence on excitation, wavelengths between 280 and 460 were verified and the behaviour was shown to be dependent.

In this study, the fixed excitation wavelength was set on 350 nm, which was when the maximum fluorescence was observed (emission wavelength was approximately 440 nm), confirming the value close to the wavelength referring to the absorption peak. The fluorescence performance is dependent on the excitation considering the fluorescence intensity, but the wavelength corresponding to the peak is not changed.

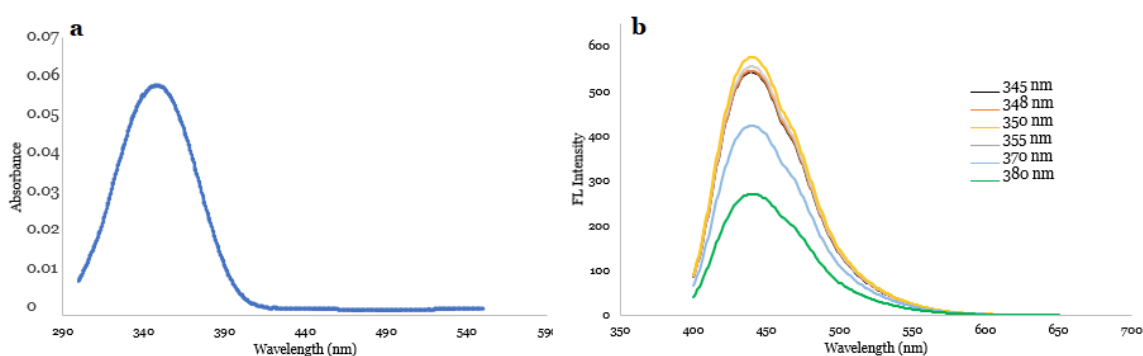


Figure 6: a) UV-Vis absorption of CDs and b) excitation-dependent FL of CDs.

### 3.1.1. Quantum yield

The entire process of optimizing the synthesis of carbon nanoparticles was based on the results of fluorescence quantum yield. The fluorescence quantum yield of the synthesized CDs was calculated from the solution of quinine sulphate, a fluorophore with a wide emission region (29) prepared in 0.1 M sulfuric acid as a reference (QY = 54%) (67), according to equation (2):

$$QY_S = QY_{ST} (I_S / I_{ST}) (A_{ST} / A_S) (\eta_S / \eta_{ST})^2 \quad \text{Equation (2)}$$

In equation (2), “ $\eta$ ” represents the refractive index, “I” the integrated fluorescence intensity and “A” the absorbance. The subscripted letters S and ST refer to the solution of the synthesized carbon dots and the quinine sulphate standard, respectively. Absorbance measurements were controlled at values equal to or less than 0.1 so that self-absorption effects were avoided and interfered with the calculations. Therefore, the QY calculations were performed using a comparative spectrophotometric method and integrating the fluorescence emission spectra in the 400–650 nm region. The excitation wavelength was 350 nm.

The characteristic absorption and fluorescence spectra of the carbon dots produced, and the quinine sulphate solution are shown in figure 7, where the maximum absorption in the UV-Vis wavelength range corresponds to approximately 350 nm.

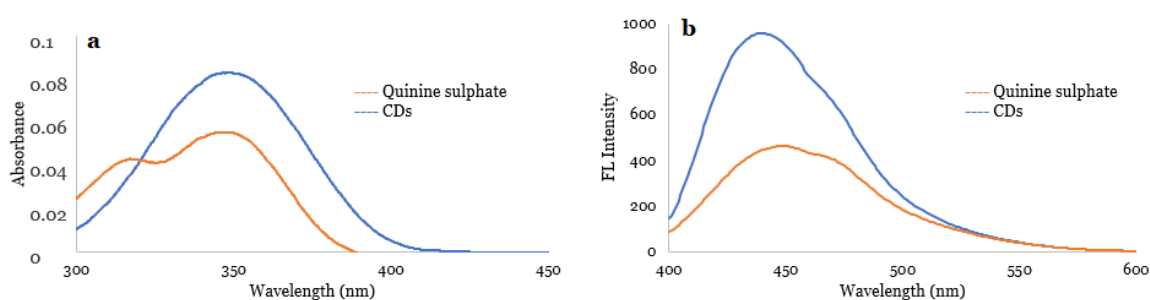


Figure 7: a) UV-Vis absorbance spectrum and b) fluorescence emission spectrum at  $\lambda_{ex} = 350$  nm, of quinine sulphate and CDs.

### 3.1.2. Optimization of CDs synthesis conditions

With the purpose of obtain the highest quantum yield of the synthesized carbon dots, an optimization was performed to establish the most suitable conditions. Different conditions for the synthesis parameters were tested, changing the reaction temperature, reaction time, microwave power and molar ratios between the reagents, by changing the volume of the nitrogen source (EDA), since the volume used of CA was fixed (3.5 mL).

Initially, an experimental design was conceptualized (Table 2) involving the study of the influence in the CDs QY of the reaction time (5, 20 and 35 minutes), the temperature for the reaction (100, 125 and 150 °C), the microwave power (30, 60 and 90 W) and the molar ratio of CA/EDA (0.6, 1.13 and 1.67). Considering the number of experiments to be performed, this initial study was divided into three days (3 blocks) of synthesis. The obtained QY results can be consulted in the table 2 and the respective photographs of the obtained CDs suspensions showing their respective fluorescence under 365 nm UV light can be seen in figure 8.



Table 2: Conditions of the first experimental design aiming at the optimization of the synthesis of CDs and obtained results (QY (%)).

Day	Run	Ratio CA/EDA	Power (W)	Temperature (°C)	Time (min)	QY (%)
1	1	1.67	30	100	05	0.74
1	2	0.60	90	100	05	0.85
1	3	0.60	30	150	05	66.64
1	4	1.67	90	150	05	67.40
1	5	0.60	30	100	35	8.42
1	6	1.67	90	100	35	2.17
1	7	1.13	60	125	20	66.99
1	8	1.13	60	125	20	73.81
1	9	1.13	60	125	20	66.64
2	10	1.67	30	150	35	61.39
2	11	0.60	90	150	35	58.53
2	12	0.60	30	100	05	-
2	13	1.67	90	100	05	4.71
2	14	1.67	30	150	05	50.86
2	15	1.13	60	125	20	53.72
2	16	1.13	60	125	20	55.81
2	17	1.13	60	125	20	59.10
3	18	0.60	90	150	05	56.31
3	19	1.67	30	100	35	1.74
3	20	0.60	90	100	35	2.44
3	21	0.60	30	150	35	64.31
3	22	1.67	90	150	35	67.88
3	23	1.13	60	125	20	62.99
3	24	1.13	60	125	20	53.90
3	25	1.13	60	125	20	60.48

From the results of the experimental design, was possible to observe that the lowest QY results (between 0.74 and 8.42 % in the table 2) were obtained in the syntheses with a temperature of 100 °C and the highest ones (more than 50%) mainly at 150 °C, demonstrating that temperature is an essential factor influencing the QY values obtained. The syntheses with low QY obtained also showed a visible reduction in their observable fluorescence under 365 nm UV light. Published in 2019, in the work from Ma. Ivy S. Dela

Cruz et al (68), that prepared CDs from polyurethane and studied the ideal conditions for synthesis with an optimization, temperature was reported as the most important factor, being responsible for controlling the processes of hydrolysis and carbonization of polyurethane. According to Mohammed Abdullah Issa et al (29), in the investigation carried out and published in 2020, where they synthesized N-doped carbon nanoparticles from carboxymethyl cellulose (CMC) and linear polyethyleneimine (LPEI), in the optimization steps, they concluded that high temperature was essential for the polymerization reaction of CMC occurred, since at low temperatures the formed solutions were transparent, while the fluorescent solutions produced were possible by the necessary carbon nuclear growth.



Figure 8: Syntheses obtained in the first experimental design under UV light at 365 nm.

Based on this first study, in which temperature had initially a more significant effect on the synthesis, a second experimental design was planned (Table 3), where the temperature was fixed at 150 °C, corresponding to highest QY previously obtained (more than 60%). The same times and power conditions were tried and the molar ratios values of 0.5, 1.0 and 2.0.

Table 3: Conditions of the second experimental design aiming at the optimization of the synthesis of CDs and obtained results (QY (%)).

Run	Ratio CA/EDA	Power (W)	Time (min)	QY (%)
1	2.00	90	35	68.05
2	2.00	30	05	62.92
3	0.50	90	05	44.15
4	0.50	30	35	45.97
5	1.00	60	20	70.46
6	1.00	60	20	68.97
7	1.00	60	20	69.41
8	2.00	90	05	61.96
9	2.00	30	35	68.89
10	0.50	90	35	52.20
11	0.50	30	05	42.73

Afterwards, the other parameters were analysed, and the results found are expressed in figure 9. Regarding the independent variables, time ( $t$ ) and power ( $P$ ) presented high QY values in all three tested possibilities, while the ratio showed more consistent values according to each condition evaluated. The 0.5 ratio had the lowest QY and the 1.0 ratio the highest QY, concluding that in principle, the ratio would be the only factor with influence. Using multiple linear regression, the molar ratio CA/EDA ( $R$ ) is the only significant variable, although only 59% of the observed variations are justified, but the existence of other significant interactions was also analysed.

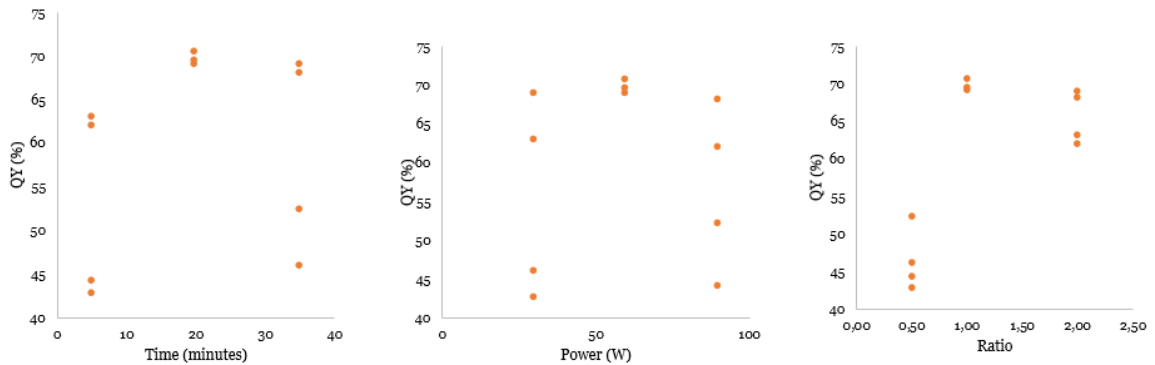


Figure 9: QY variation (%) as a function of the independent variables time, power, and ratio.

When including possible interactions between the variables, it was observed that the greatest influence is time ( $t$ ), time x time ( $tt$ ) and ratio ( $R$ ). The contribution of the power-ratio ( $PR$ ) interaction is negligible ( $p$ -value  $< 0.05$ ). The final mathematical model obtained, excluding the non-significant interaction  $PR$  is found in equation (3), with R-squared = 99%.

$$QY (\%) = 69.6133 + 2.91875 * t + 9.59625 * R - 13.7546 * tt \quad \text{Equation (3)}$$

Then, to confirm the model obtained, a set of experiments involving experimental conditions whose predicted QY, accordingly with the previous mentioned mathematical model, were approximately 60, 65, 70 and 75% was planned and are shown in table 4. Experimental conditions  $t$  and  $R$  were rounded to values that are easy to manipulate in the laboratory.

Table 4: Parameters for validation of the optimized model of CDs synthesis conditions and QY (%) results obtained (Temperature = 150 °C, Microwave Power = 60 W).

Run	Time (min)	Ratio	QY (%)	Predicted QY (%)
1	20	0.50	49.47	60.0
2	20	0.50	51.35	60.0
3	20	0.80	63.37	63.9
4	20	0.80	62.94	63.9
5	20	1.25	67.17	69.6
6	20	1.25	67.54	69.6
7	10	0.90	67.90	57.1
8	10	1.40	65.60	63.5
9	10	2.25	58.72	74.3

Through this test it was observed some correlations between the observed QY and the predicted QY for the experiments conducted in the time of 20 minutes, but at 10 minutes the correlation is different from what was expected, mainly in the ratio equal to 2. As new ratios were tested, the QY under these conditions were different from predicted, which may be indicative of previously unverified interactions of time and ratio or of other greater magnitude. In summary, of the 9 confirmation tests performed, 4 results were considered anomalous or deviating from what was expected, namely the experiments run 1, run 2, run 7 and run 9. It is important to mention, that the experiments run 1 and run 2 corresponded to a combination of variables in an experimental space that was not tested before, being the predicted results affected by the low prediction capability of the model in these cases.

Redoing the analysis with only the 6 experiments involving a synthesis time of 20 minutes and adding the results obtained in the optimization described in the table 4, it is obtained the graph of figure 10, which allows us to conclude as more significant the same variables  $t$ ,  $tt$  and  $R$  identified in the previous experimental design, reinforcing thus the obtained results. The new mathematical model obtained was statistically similar, with an R-squared of 91%, as shown in equation (4), where  $t$  is the time and  $R$  is the molar ratio of CA/EDA.

$$QY (\%) = 69.61 + 2.919*t + 9.60*R - 13.76*tt \quad \text{Equation (4)}$$

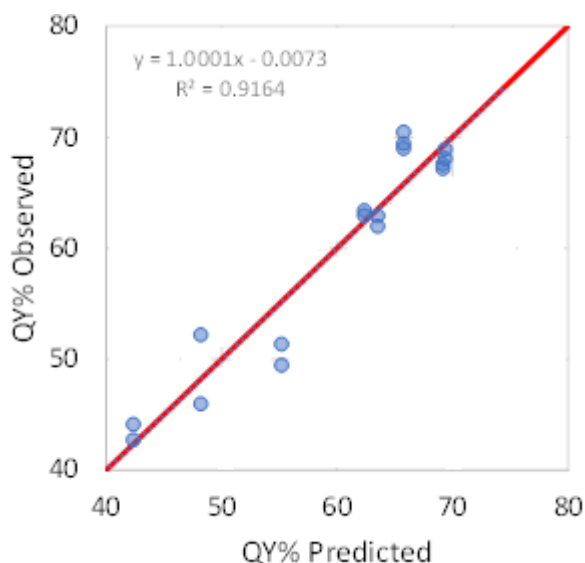


Figure 10: Comparison of the observed and predicted QY of the confirmation model.

It is interesting and valid to emphasize, however, that run 7 and run 9 showed results "opposite" to what was expected, and even performing repetitions, the effect obtained experimentally was the same. It was not possible to conclude the causes that justify the atypical behaviour. By including in the previous mathematical model, the observed result of the assay run 8, since this result was in accordance with the predicted QY value, the final model obtained, with a R-squared equal to 91.74% is represented in equation (5).

$$QY (\%) = 65.8268 + 2.84252*t + 10.6347*R - 9.91723*tt \quad \text{Equation (5)}$$

Continuing with the optimization of the synthesis of CDs, it was evaluated the possibility of further maximize the QY already obtained and based on the last mathematical model validated in the multivariate region comprised between  $5 < t < 35$  min,  $0.5 < R < 2.0$ ,  $T = 150$  °C and  $P = 60$  W. By analysing that model, it was hypothesized the maximization of the response QY, by increasing the molar ratio  $R$ , since it presented a positive coefficient in the model and by increasing the  $t$ . The time, however, cannot be increased too much due to the negative coefficient of  $tt$  observed in the mathematical model and from the energetic point of view, given that it is desirable to minimize the CDs synthesis time. Accordingly, a QY of 80% was predictable using  $R = 2.5$  ( $T = 150$  °C,  $t = 20$  min,  $P = 60$  W) and a QY of 90% using  $R = 4.9$  ( $T = 150$  °C,  $t = 20$  min,  $P = 60$  W). However, the expansion of the  $R$  range lacks experimental validation as only the region between 0.5 and 2.0 was already studied.

Nevertheless, an experimental plan was established considering the possibility of further enhancing the QY, and thus, the experimental conditions tested and respective observed QYs are described in the table 5.

Table 5: Parameters for maximizing QY (%) and the obtained results (T = 150 °C, P = 60 W).

Run	Time (min)	Ratio	QY (%)	Predicted QY (%)
1	20	1.00	68.8	65.8
2	20	2.00	69.9	76.5
3	20	4.00	48.6	87.1
4	10	1.00	68.4	59.5
5	10	2.00	63.0	70.2
6	10	4.00	40.7	80.8

Based on the last experiments performed, and by also including the assays and observed results included in the table 5 (excluding the previous identified anomalous results), it was possible to obtain an R-squared of approximately 98% related to the mathematical model exposed in equation (6).

$$QY (\%) = 67.8688 - 5.10191 * R - 17.8941 * RR + 4,69045 * tR \quad \text{Equation (6)}$$

The coefficient of  $t$  in the model indicates that the shorter the time, the greater should be the QY%. However, experiments carried out previously with a time of 5 minutes, did not yielded results of QY higher than 63%. This may be justified considering there is an optimal time for the CDs to be formed, and then the yield decreases.

Finally, an overall compilation of all the verified synthesis and corresponding results, comprising the reaction times between 5 minutes and 35 minutes and the molar ratios between 0.5 and 4.0, allowed to achieve a R-squared of 93% for the final mathematical model, conform equation (7). The graph in figure 11 is relative to the times from 10 to 35 minutes and ratios from 0.5 to 4.0 and the respective QY obtained.

$$QY (\%) = 70.6666 + 2.80758 * t - 2.26231 * R - 2.62548 * tt - 23.1552 * RR \quad \text{Equation (7)}$$

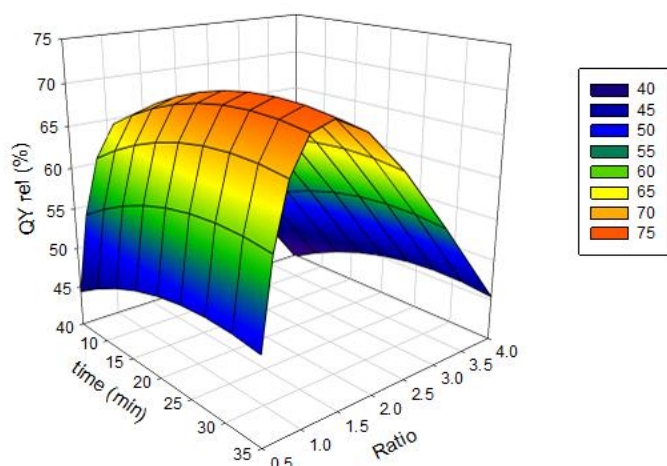


Figure 11: Influence of the synthesis time and reagents ratio on the CDs QY (%).

In conclusion, the experimental conditions in the CDs synthesis, that allowed to obtain the highest quantum yield of approximately 70%, while using the minimum reaction time, and that were used to proceed with the present work, were: temperature of 150 °C, power 60 W, molar ratio CA/EDA of 1:1 and reaction time of 10 minutes. In figure 12 there is an image of the respective syntheses of the last experiment (Table 5) and the absorbance and fluorescence spectra characteristics of the CDs solution under these conditions are shown in figure 13.

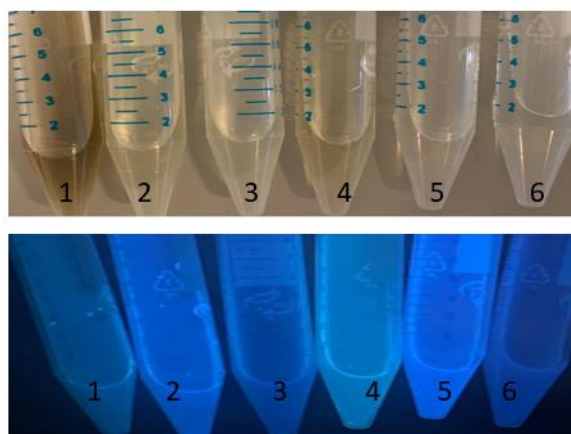


Figure 12: Photographs of CDs syntheses (Table 5) under ambient light (above) and under UV light at 365 nm (below).

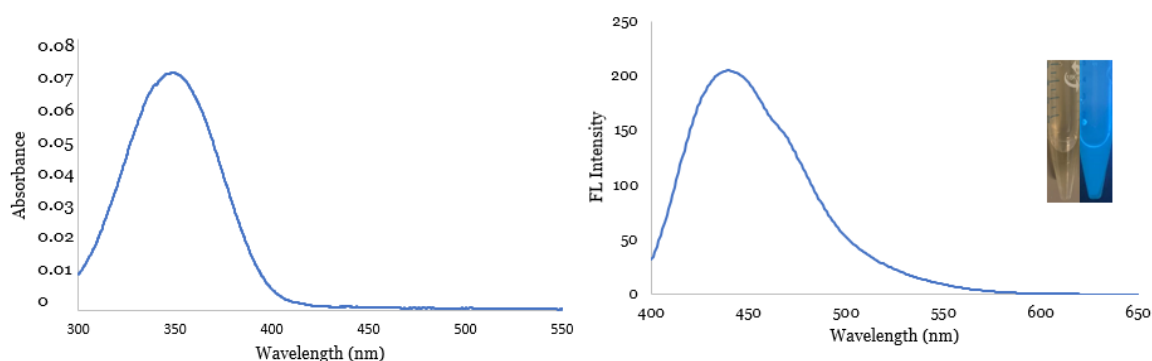


Figure 13: Absorption and fluorescence spectra ( $\lambda_{\text{ex}} = 350 \text{ nm}$ ) of CDs synthesis under optimized conditions.

### 3.2. Preliminary studies of detection of nitrite ions

After obtaining the experimental conditions that allowed to synthesize CDs with approximately 70% QY, in only 10 minutes, some CDs suspensions were prepared and submitted to lyophilization. The obtained dry material was weighed to allow the calculation of a rough concentration value upon dispersion of the lyophilized CDs in a determine volume of ultra-pure water. Then, using the calculated concentrations of the CDs stock solutions, some dilutions were prepared, to allow the execution of preliminary tests for detection of nitrite ions. The detection procedure was based on the fluorescence quenching of carbon dots caused by its reaction with nitrite ions. The fluorescence quenching of the CDs solution by the addition of nitrite ions can be analysed by the following Stern-Volmer Equation (8):

$$F_0/F - 1 = K_{sv} [C] \quad \text{Equation (8)}$$

Where “ $F_0$ ” and “ $F$ ” correspond to the fluorescence intensity of the CDs without and with the addition of the nitrite solution, respectively. “ $K_{sv}$ ” is the extinction constant, which corresponds to the slope of linearity and “[ $C$ ]” is the concentration of the nitrite solution.

Based on the article published by Lanlan Gan et al in 2020 (66), a stock solution of nitrite ions at a concentration of 0.01000 mol/L was prepared from 345.0 mg of sodium nitrite and addition of water up to a volume of 500.0 mL in a volumetric flask. A fixed volume of 150  $\mu\text{L}$  of the CDs solution and 6 different  $\text{NO}_2^-$  volumes (10, 20, 50, 100, 150 and 200  $\mu\text{L}$ ) were established to add to a 100 mL volumetric flask and topped up with water. It was measured the respective fluorescence intensities for comparison with the fluorescence intensity without the presence of nitrites (sample blank), as shown in table 6. The excitation wavelength was set at 350 nm, and the monitored emission wavelength was 440 nm.



Table 6: Fluorescence conditions and results obtained from the reaction of different concentrations of nitrites with CDs at a concentration of 82 mg/mL.

Run	NO <sub>2</sub> <sup>-</sup> (mg/L)	NO <sub>2</sub> <sup>-</sup> (μL)	FL Intensity	(F <sub>0</sub> /F) – 1
Sample blank (Control F <sub>0</sub> )	0	0	900.2	0
1	98.6	10	873.5	0.0305
2	197.1	20	846.3	0.0637
3	492.8	50	844.6	0.0658
4	985.6	100	640.8	0.4047
5	1478.5	150	565.8	0.5911
6	1971.3	200	501.1	0.7965

The previous obtained results were used to assess the linear response range, resulting in the graph shown in figure 14, which presented a R-squared of 0.977.

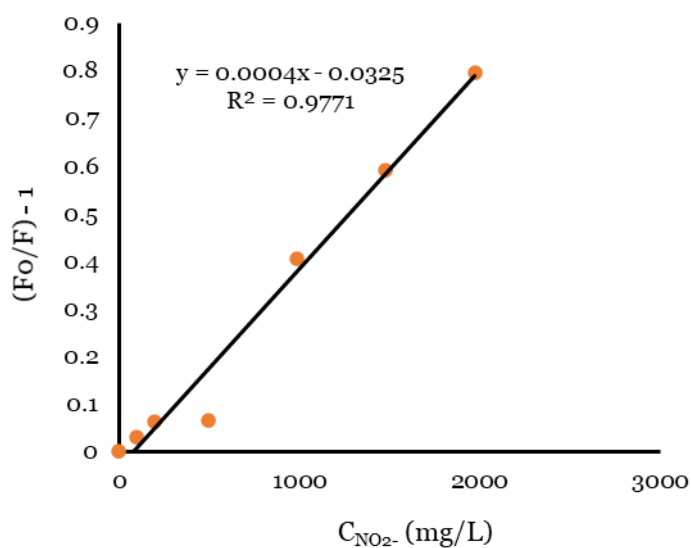


Figure 14: Study of the linear response range between FL quenching ((F<sub>0</sub>/F) – 1) and the concentration of nitrite ions (C<sub>CDs</sub> = 82 mg/mL).

It was possible to conclude at first that the fluorescence quenching occurs under the conditions evaluated, but the nitrite concentrations tested were too high for practical determination applications.

### 3.2.1. Study of the influence of the concentration of CDs and reaction time

A new design of experiments was carried out, establishing a lower concentration of nitrites. With the nitrites concentration of 1 ppm fixed, an analysis of the influence of the concentration of CDs on the reaction was carried out. From a CDs stock solution of 87.88 mg/mL, it was prepared a solution of concentration 0.1 mg/mL, from which different volumes were varied to assess the impact of the CDs concentration on the detection of nitrites.

The proposed parameters are shown in table 7, where the concentration of CDs was varied from 0.001 mg/mL to 0.055 mg/mL. For each test a sample blank measurement (without addition of nitrite), and two measurements with nitrite samples were made. The measurements in the fluorescence spectrophotometer with the addition of nitrites were carried out twice, the second with an interval of 5 minutes to verify if the reaction time influenced the result.

Table 7: Parameters and tests to study the influence of CDs on nitrite detection.

	<b>CDs (mg/mL)</b>	<b>NO<sub>2</sub><sup>-</sup> (ppm)</b>	<b>FL Intensity</b>	<b>FL intensity after 5 minutes</b>
1	0.001	1	Insignificant intensity	-
Sample control 1	0.001	0	Insignificant intensity	-
2	0.005	1	Insignificant intensity	-
Sample control 2	0.005	0	Insignificant intensity	-
3	0.01	1	119.4	117.9
Sample control 3	0.01	0	115.5	-
4	0.02	1	240.8	218.9
Sample control 4	0.02	0	236.4	-
5	0.03	1	370.3	348.2
Sample control 5	0.03	0	372.6	-
6	0.04	1	489.0	477.7
Sample control 6	0.04	0	498.2	-
7	0.05	1	602.4	582.2
Sample control 7	0.05	0	600.2	-
8	0.055	1	684.1	658.7
Sample control 8	0.055	0	682.0	-

It was found that after 5 minutes the FL intensities decreased and showed more coherent values compared to the control values, where the presence of nitrites caused a decrease in the fluorescence intensity value, as expected.

At concentrations of 0.001 and 0.005 mg/mL of CDs, the fluorescence intensity was negligible and hence, the monitoring of the fluorescence quenching was not possible. At the concentration of 0.01 mg/mL of CDs, the presence of nitrites did not show a significant influence, and the results were very similar, even after 5 minutes. Considering only the measurements after 5 minutes, in the range of CDs concentrations between 0.02 and 0.055 mg/mL, the results were consistent, and the values obtained in relation to quenching showed to being indifferent to the concentration of CDs. This conclusion is consistent with the study conducted in 2016 by Haimin Zhang et al (69): N-doped CDs were obtained for nitrite detection and found that a reaction time of at least 5 minutes was necessary to reach the stability of fluorescence intensity.

After verifying the need for the reaction time to observe the fluorescence quenching, different time intervals (0, 5, 10, 15, 20, 25, 30 and 35 minutes) were tested for a better understanding of the effect, that is, it was conducted a study of the influence of the reaction time, between CDs and nitrite ions, in the final fluorescence quenching, using for that purpose, a fixed concentration of 0.02 mg/mL CDs and 1 ppm of nitrites solution. The sensitivity of the equipment was set at 400 V to obtain fluorescence intensities in the range of 600–700. The assays were repeated three times, and the fluorescence quenching was again evaluated through the comparison of the signal obtained by the samples and the sample blanks. The conditions and results are contained in table 8 and the test graph in figure 15.

Table 8: Experimental conditions of assays for the study of the influence, in the fluorescence quenching of CDs, of reaction time between nitrite ions and CDs, and obtained results.

Run	Time (min)	FL Intensity	$(F_0/F) - 1$
Sample blank (Control $F_0$ )	-	645.8	-
1	0	726.7	-0.1113
2	5	690.5	-0.0647
3	10	661.1	-0.0232
4	15	615.4	0.0493
5	20	584.7	0.1044
6	25	549.5	0.1752
7	30	545.2	0.1844
8	35	556.2	0.1610

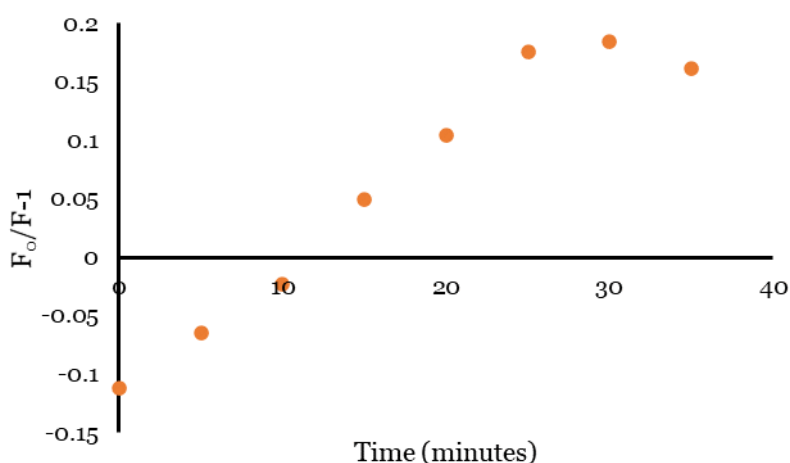


Figure 15: Influence of reaction time between nitrite ions and CDs in the fluorescence quenching of CDs.

By analysing the results, it was possible to conclude that up to 10 minutes of reaction the results were not completely reliable since it was not possible to verify the quenching until this time interval. After 15 minutes, all assays showed fluorescence quenching in relation to the control sample, being even more reduced after 25 minutes. Additionally, it could be observed that after 25 minutes of reaction, the fluorescence quenching values tended for stabilization. Thus, the obtained results indicate 25 minutes as the optimum time for the reaction between CDs and nitrites considering the objective of the proposed work. In figure 16, there is a comparison between the FL spectra of the control sample and the sample with nitrites with a reaction time of 25 minutes.

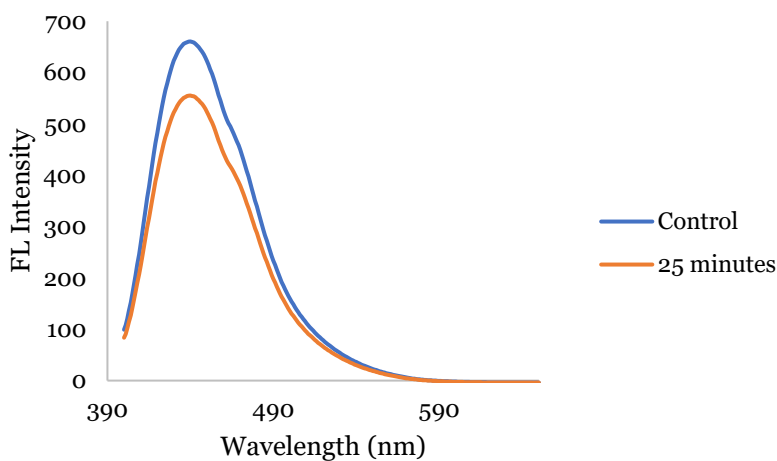


Figure 16: Fluorescence spectrum of carbon dots (0.02 mg/mL) with nitrite ions (1 ppm), after 25 minutes of reaction time.

After confirming the need for 25 minutes of reaction time for the detection of nitrites using N-doped CDs, the influence of CDs concentration was re-examined. For that purpose, a set of experiments, represented in the table 9, was executed. A sodium nitrite solution was used with a fixed concentration of 1 ppm and a variable concentration of CDs from 0.005 to 0.05 mg/ml. For each concentration of CDs tested, the fluorimeter reading of the control sample was also taken, without the addition of nitrites. The complete test was repeated three times (divided into three different days) and the results obtained can be consulted in table 9.

Table 9: Experimental conditions, and obtained results, of assays for the study of the influence in the fluorescence quenching of CDs, of different concentrations of CDs (0.005 – 0.05 mg/mL) reacting with 1 ppm of nitrite ions (reaction time fixed at 25 minutes).

CDs (mg/mL)		Day 1	Day 2	Day 3	FL quenching <sup>(a)</sup>
0.005	C1	658.764	649.486	641.262	0.013
	F1	652.614	637.189	633.798	
0.01	C2	726.677	733.451	710.837	0.037
	F2	721.904	664.340	706.670	
0.02	C3	775.462	782.827	759.803	0.080
	F3	724.690	703.952	718.666	
0.03	C4	812.749	866.708	837.322	0.049
	F4	783.289	832.388	784.656	
0.04	C5	858.468	842.517	872.841	0.029
	F5	833.592	831.122	835.385	
0.05	C6	888.869	874.402	863.486	0.020
	F6	856.219	868.640	849.507	

C<sub>1</sub> – C<sub>6</sub>: sample control (without analyte) of runs 1 – 6; F<sub>1</sub> – F<sub>6</sub>: sample (C<sub>NO<sub>2</sub><sup>-</sup></sub> = 1 ppm) of runs 1 – 6; <sup>(a)</sup> fluorescence quenching calculated using the equation  $(F_0/F) - 1$  (averages of C<sub>1</sub> – C<sub>6</sub> and F<sub>1</sub> – F<sub>6</sub> were used).

By observing the results of the table 9, it was verified an increase of the FL intensity as the concentration of CDs increases from C<sub>1</sub> to C<sub>6</sub>, as expected. However, the same tendency in the fluorescence quenching was not observed. In fact, there is a higher mean fluorescence quenching at the CDs concentration of 0.02 mg/mL (Figure 17). Consequently, among the concentrations evaluated, a CDs concentration of 0.02 mg/mL is advisable to be used in future tests due to the better result obtained.

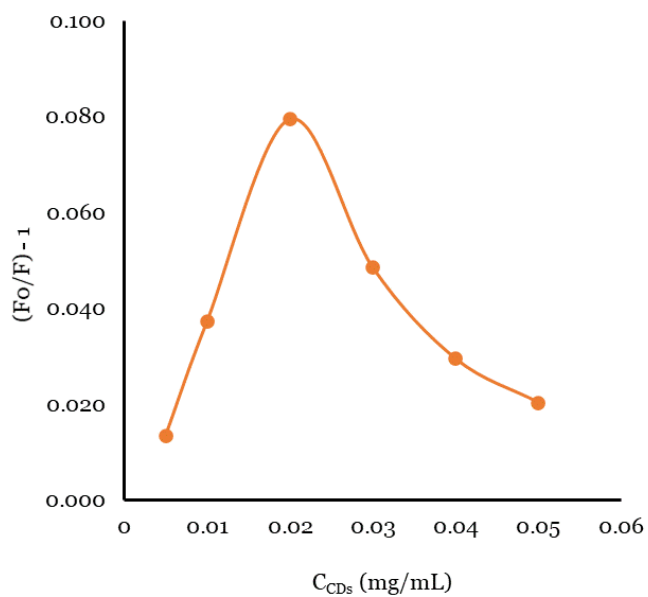


Figure 17: Graphical representation of the variation of fluorescence quenching of different concentrations of CDs, when reacting with nitrite ions at a fixed concentration of 1 ppm.

## 4. Conclusions and future work

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The determination of nitrite ions is an important parameter across the health, food, and environmental areas. Human contact with these ions' crosses both intrinsic and extrinsic fonts, but the food pathway is considered the main source of this contact, given the use of nitrite salts in food products. Despite the non-nanotechnological methods for detecting the presence of nitrite ions currently existing and even certified, possible new approaches for a simpler, cheaper, and more efficient analysis among those already published continue to be studied and nanomaterials emerge as chemically attractive compounds to be modifying agents associated with known methodologies. Within the scope of carbon-based nanomaterials, carbon dots nanoparticles have special relevance due to their growing use in the development of new methodologies for the determination of nitrites by fluorescence-based sensors.

Based on the experiments in the present work, it could be concluded that it was possible to successfully synthesize the fluorescent carbon dots, they had optical characteristics consistent with other similar studies reported in the literature and with a high quantum yield, using a fast and green synthesis method. CDs produced shown to be sensitive to the reaction with nitrites, even without external adjustments such as a change in pH or temperature for detection. However, preliminary tests performed were not sufficient to determine a linear range of possible detectable nitrite concentrations, nor a detection limit. The developed work allowed to confirm that a minimum of reaction time is strictly needed to stabilize the fluorescence intensity relative to the reaction between nitrites and carbon dots, as well as an ideal concentration of CDs present.

For future work, further investigations are needed about the performance of carbon dots, tests at different pH values and the interference of other ions. The associated advantages that inspire a continuity in the investigation of carbon dots synthesis and application in the detection of nitrites are the low required amounts of reagents, fast synthesis time and easy execution, which could become a promising method in the future. The use of CDs for the detection of  $\text{NO}_2^-$  in food samples is still an area to be explored and more studies still need to be done to ensure the reported sensitivities, feasibility, and reproducibility of the techniques, especially in real samples, which can be complex and varied.



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