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Original Research

Passive micromixer integration with a microfluidic chip for calcium assay based on the arsenazo III method

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Abstract The ability for low reagent consumption and minimum waste production in a miniaturised system has generated great interest in the green chemistry field. Herein, a microfluidic system for calcium assays using the arsenazo III method has been developed. The reaction between arsenazo III and calcium to form a blue-purple coloured complex is measured by an embedded miniature fibre optic spectrometer through absorbance increments at 650 nm. A linear range was obtained from 0.2 to 3 mg dL^{-1} with a detection limit of 0.138 mg dL^{-1} (S/N=3). The method exhibited good reproducibility based on low and high calcium tests with control serums, the within-run coefficient of variation (CVs) (4.10% and 3.91%), and the run-to-run CV (4.6%) were obtained. The carry-over effect of the method was also 1.98%, which is acceptable for the current system. When compared to a conventional spectrophotometric method, this portable, microfluidic method correlated highly when evaluating serum samples (r^2=0.985; n=15). This similarity suggests that our proposed system could be used for determining the amount of calcium in serum samples.

Keywords: Arsenazo III, Calcium determination, Portable microfluidic system, Passive micromixer

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Introduction

The calcium ion plays an important role in blood coagulation, neuromuscular conduction, maintenance of membrane function, and control of skeletal and cardiac muscle contractility[1]. Increased calcium levels in serum are reported in primary hyperparathyroidism, hyperthyroidism, and malignancy, whereas decreased levels are observed in primary hypoparathyroidism, acute pancreatitis, and vitamin D deficiency[2]. In other words, the determination of calcium levels is very useful for clinical diagnosis.

Specific dye binding methods, such as methylthymol blue[3], o-cresolphthalein complexone[4,5], and arsenazo III[6,7] are often used for determining total calcium levels because of their rapidity, convenience and inexpensiveness. However, the method utilising methylthymol blue or o-cresolphthalein complexone suffers from dye instability in an aqueous solution. Therefore, the reagent kit must contain two separate compositions. Arsenazo III is chemically stable and has a very high affinity for calcium over a wide pH range and it can be prepared and used as a single assay reagent[8]. Consequently, the arsenazo III method has become more popular. Nevertheless, arsenazo III is an organic, arsenic compound that contributes to environmental problems. Because the goal of green chemistry is to minimise reagent consumption and waste production[9], one possible solution to this problem involves microfluidic-based platforms. Microfluidics is known for low reagent consumption and lower waste production. There-

fore, by combining a microfluidic system and the arsenazo III method, a reduction in arsenic's environmental effect should occur.

For this study, a portable microfluidic system based on the arsenazo III method was configured for assaying calcium. Arsenazo III reacts with calcium to form a blue-purple coloured complex, which is measured by the integrated miniature fibre optic spectrometer with a 650-nm absorbance increment. In microfluidic-based biochemical analysis, homogeneous mixing is crucial for most bio-analytical systems to obtain high sensitivity and good reproducibility. Typically, with the small dimensional channel of a microchip, complete mixing will only occur when the two fluids are dispersed with each other in the decimetre lengths of a microchannel[10-12]. As a result, micromixer designs for integration into Lab-on-a-chip (LOC) technology have made great strides to reduce the need for long mixing lengths[13,14]. Passive, planar micromixers utilising various obstacles for fluidic and particle mixing, have became an alternative approach[15,16]. They can be easily fabricated and integrated into microfluidic systems.

In our study, the J-shaped obstacles were modified to exaggerate the mixing effect; four sets of 10-piece baffles were introduced within the main microchannel. Herein, a computational simulation was performed with our micromixer, and its mixing results were compared with those obtained from an experimental fluorescence technique. The two approaches showed similar results, which indicated the versatility of the proposed passive micromixer and supported its possible integration into a LOC system. Moreover, the applicability of the micromixers for sample analyses with calcium evaluated the effectiveness of the proposed system.

Results and Discussion

Simulation and evaluation of mixing efficiency

Although a similar microchip-based system has been implemented in our group[17,18], the fluorescence experiment to examine the mixing capability had yet to be investigated. To verify the results acquired from the fluorescence image acquisitions, it had to be assured that the mixing results would be identical to those of the computational simulation. If those could be affirmed, our proposed microfluidic chip would pose to be a valuable model for clinical analysis. The passive microfluidic mixer with J-shaped baffles in the T-channel was first put forward by Yu-Cheng Lin *et al.*[19]. They asserted that their design was particularly suitable for on-chip blood analysis. However, the application for on-chip biochemical analysis was not yet fully exploited. Therefore, the T-channel with a modified J-shaped structure was employed for our mixing purpose.

According to the simulation, it was clearly indicated that almost complete mixing was achieved after the solution passed through four mixing sets of J-shaped baffles; in other words, its contents became gradually homogeneous, as shown in Figure 1A. When comparing the simulation results with the fluorescence experiment, the mixing performances were quite similar (Figure 1B), as indicated by the intensity of the converted grey scale for the fluorescent signals.

In Figure 1C, the mixing percentage between the simulation and the fluorescence results are represented. Our proposed design enhanced the mixing of the two fluids by introducing lateral convection in the microfluidic channel. In addition, baffles within the microchannel strongly encouraged chaotic flow. As a result, the fluid distributions gradually became homogeneous as the downstream mixing-sets increased. Additionally, a homogeneous mixing was attained in a short time and within a limited length; a mixing percentage of $\sim$90% homogeneity was obtained after passing all mixing regions, which accounted for a 25-mm length.

Linearity

Under the proper condition, the proportional relationship between the concentration of calcium and the analytical signal was plotted to produce an analytical curve. The linear range was obtained from 0.2 to 3 mg dL^{-1} of calcium (r^2=0.996) (data not shown), and the detection limit was found to be 0.138 mg dL^{-1} or 0.0345 mM (S/N=3), respectively. Our proposed method is slightly more sensitive than the method described previously by N. Malcik *et al.*[20]; their detection limit is 0.085 mM. Additionally, the sensitivity of this system is similar to another method that used reflectance measurements of arsenazo III immobilised polymer beads[21]; however, its detection limit is maximised at 0.0268 mM.

Effect of pH and temperature

In this research, an alkaline pH region was selected for measuring calcium levels because the arsenazo III method sensitivity increased when compared to the reaction occurring in an acidic region. Hence, boric acid/sodium hydroxide solutions were used to maintain the pH in the range of 8, 8.5, and 9. The optimal concentration of arsenazo III was prepared with three different pHs of 25 mM boric acid buffer containing 5 mM 8HQS. Under the alkaline environment, the 8HQS binds to magnesium; thus, it competes with calcium

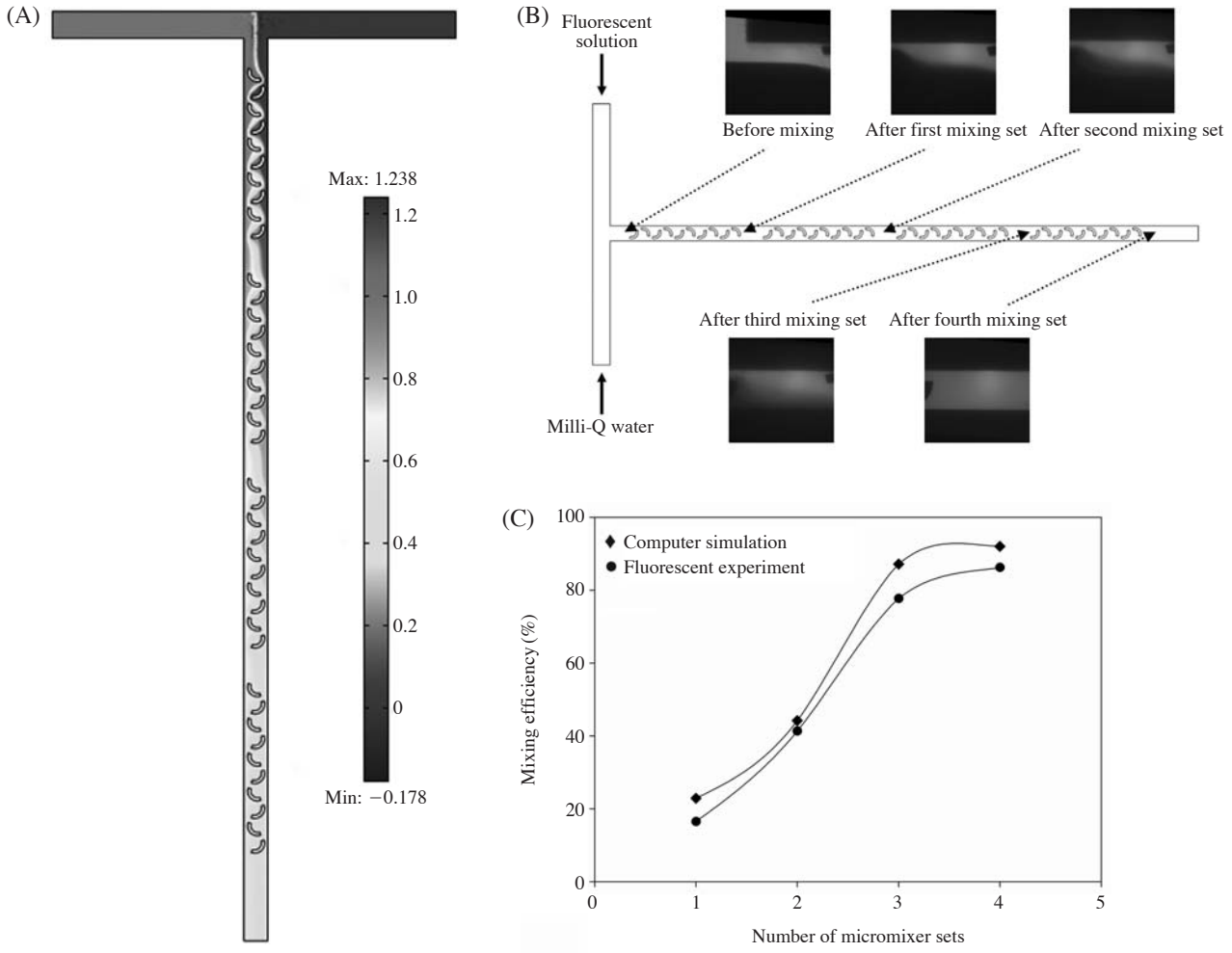

Figure 1. Comparison of the microchip mixing performance at the flow rate of 40 μL min^{-1} (Re=3) as evaluated by (A) a computer simulation and (B) captured fluorescent images. (C) Relationship between the mixing efficiency versus the number of micromixer sets fabricated on the microchannel as assessed by A (◆) or B (●).

to form a complex with arsenazo III[7]. Although the calcium-arsenazo III complexes at pH 9 provided the highest response signals, they were disregarded due to their narrow linearity. The pH 8 buffer was instead selected for all subsequent experiments because it provided a wider linearity, and up to 3 mg dL^{-1} of calcium could be assayed (Figure 2).

To gauge the effect of temperature on the system, studies were conducted at 25, 30, 35, and 37°C. A calcium concentration of 3 mg dL^{-1} was assayed in duplicate at the four different temperatures. The temperature at 30°C was selected for further experiments because it provided the highest response signal as shown in Figure 2.

Reproducibility and carry-over effect

Two levels of control serum were assayed for calcium content to study the reproducibility of the system. The results showed that within-run reproducibility was attained at 4.10% and 3.9% CVs, respectively, when performed with low- and high-level control serums on the same day (each concentrations n=10). Moreover, run-to-run reproducibility was obtained at 4.6% CVs, as assessed on three different days (n=10). These results indicate that our proposed system provided good reproducibility.

Carry-over is commonly used to describe a process by which the reagents or samples are transferred into another reaction. In our case, the term carry-over is possibly caused by the carrying of the arsenazo III reagent into a subsequent reaction because of insufficient washing. Owing to the reusability of our microfluidic device, a carry-over effect should be considered. The carry-over effect study was performed following a method described in a previous study[22]. With our

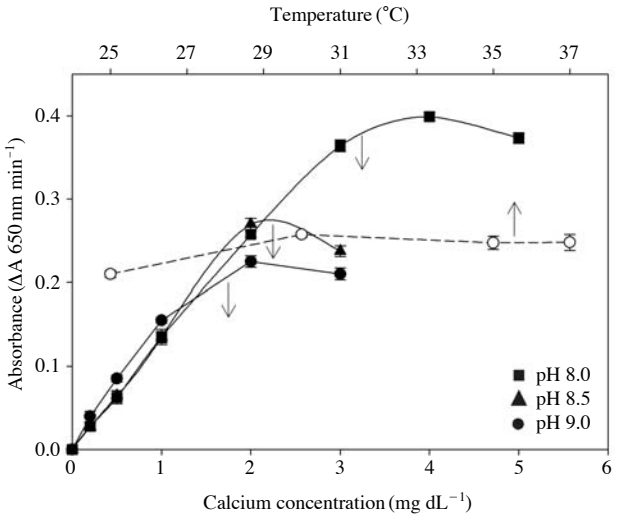

Figure 2. The effect of the carrier buffer pH (solid lines) and temperature (dash line) to the assay signals.

Table 1. Interferences effects on the measurement of calcium using the arsenazo III method.

Added interferences	Added concentration (mg dL^{-1})	%Recovery
Magnesium	123	95.3
Haemoglobin	500	83.9
	50	100.7
Bilirubin	20	81.2
	0.5	97.3
Iron	0.1	99.3
Glucose	10	103.7
Ascorbic acid	0.5	95.7
NaCl	900	96.3

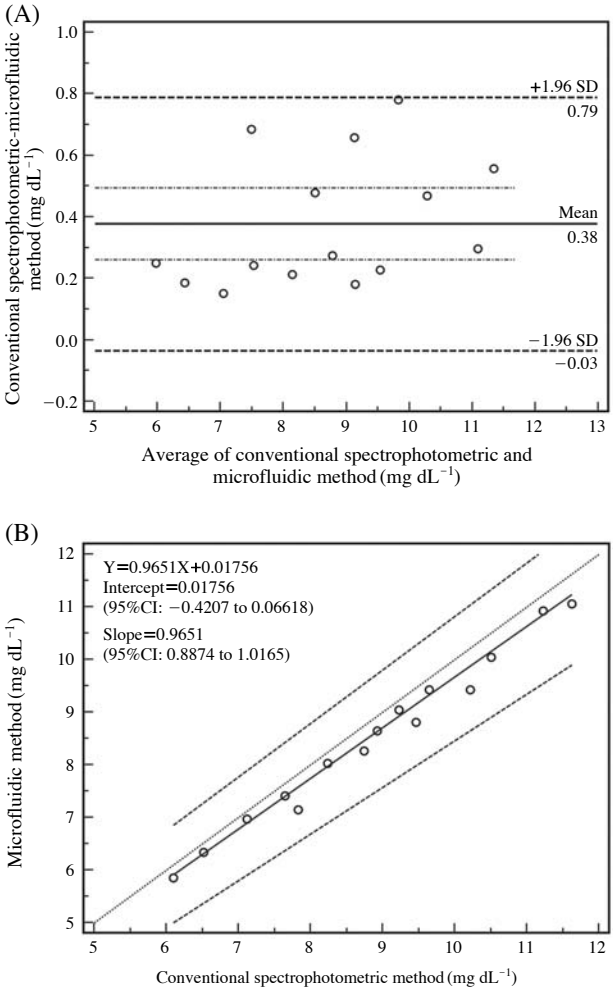

Figure 3. Comparison between the proposed microfluidic system and a conventional method using (A) a Bland-Altman bias plot and (B) a Passing-Bablok regression analysis.

method, the carry-over effect was calculated to be 1.98%, which is acceptable for the current system. Because the PDMS surface was regenerated using 1 M HCl after each assay, the carry-over effect was low to negligible. In addition, using an acid-washing step helped eliminate contaminated traces of calcium from the system before starting a new run.

Interferences

A known amount of interference was spiked into the control serum samples, and then the percentage recovery was calculated from the ratio of the initial response signals to the spiked signals. As shown in Table 1, the results revealed that the interference from magnesium, iron, glucose, ascorbic acid, and sodium chloride were acceptable with the recoveries between 95.3-103.7%. Comparatively, a haemoglobin concentration of 500 mg dL^{-1} affected the calcium assay with a recovery of 83.9%. However, when the concentration of haemoglobin decreased to 50 mg dL^{-1}, the percent of recovery became more acceptable at 100.7%. Considering bilirubin, the results displayed that 20 mg dL^{-1} could seriously interfere in the proposed assay. By contrast, 0.5 mg dL^{-1} of bilirubin was quite satisfactory, giving a recovery signal of 97.3%. Nevertheless, because bilirubin is usually low in human blood, this interference could be feasibly disregarded[2].

Assay comparison

Fifteen samples were tested for calcium content utilising our system in parallel with the current large-scale methods. Owing to the linearity of our system, ranging from 0.2 to 3 mg dL^{-1}, of calcium, the samples were

diluted with saline before analysis. To get various amounts of analysts, including those with lower and higher than normal calcium levels, the real serum samples were spiked with low or high levels of calcium from the commercial control serums before assaying.

The results were analysed with a Bland-Altman bias plot and a Passing-Bablok regression. Both the upper and lower horizontal lines had a 95% confidence interval for their limit of agreement. Furthermore, the results demonstrated a good relationship between the proposed microfluidic system and the conventional spectrometer method because they both had a mean SD ± 1.96 (Figure 3A). In Figure 3B, the regression equation according to Passing and Bablok was $y = 0.9651x + 0.01756$. According to the 95% confidence interval noted above, the plotted graph's values for the y-intercept (0.01756) and the slope (0.9651) must be significantly reliable and fell in the range of −0.4207 to 0.06618 and 0.8874 to 1.0165, respectively. In other words, these data suggest that the proposed microfluidic system is highly correlated with the conventional spectrometer method, using an assay kit from Randox.

Conclusions

Sufficiently rapid, homogeneous mixing of the assay reagent and sample was achieved within the microchannels because of the planar micromixers with J-shaped obstacles. Obviously, this strategy was potentially accelerated by the uniform mixing of both reagents in the short mixing path of the microfluidic chip. Results from the computer simulation and the captured fluorescence images were similar and indicated that the proposed micromixer would be effective for enhancing the mixing performance of the system.

With this proposed microfluidic system, the absorbance detection of calcium assays can be performed directly on-chip, while also being fully portable. From this point of view, this method proves promising to perform in field analyses of calcium from human samples or even from environmental samples. Additionally, this miniaturised system corresponds closely with the concept of green chemistry, which focuses on the reduction or elimination of toxic waste products.

Materials and Methods

Chemicals and reagents

All chemicals were analytical reagent grade. Arsenazo III, bilirubin, and fluorescein sodium salt were purchased from Fluka (Buchs, Switzerland). Boric acid 8-hydroxyquinoline-5-sulphonic acid (8HQS), haemoglobin, ascorbic acid, D-glucose, magnesium sulphate, and sodium chloride were obtained from Sigma (St. Louis, USA). Calcium carbonate was supplied by Mallinckrodt (St. Louis, USA). Iron standard, hydrochloric acid, and sodium hydroxide were purchased from Merck (Darmstadt, Germany). The calcium assay kit used for method validation was provided by Randox Laboratories (Crumlin, United Kingdom). The control serums (Liquid assayed multiqual®) from Bio-Rad Laboratories (Berkeley, USA) were intended as assay control serums to monitor the reproducibility in the studies. PDMS kits (Sylgard 184) used in the fabrication of the PDMS microfluidic device were provided from DowCorning (USA). Photoresist (SU-8 2100) and its developing reagent were supplied by Micro-Chem (USA).

Microfluidic simulation

The mixing efficiency was evaluated using 3D simulations on COMSOL software (Fluent Inc., NH, USA) to solve the conservation equations for mass, momentum, and diffusion-energy. Ethanol and water were introduced into two branches of a T-shaped microchannel. The simulation environment was verified for steady incompressible flows. The total number of cells in this micromixer was approximately 110,000 and the simulations were run for 250 iterations. To define the boundary conditions, three physical properties of ethanol and water (density, viscosity, and diffusivity) were applied in the simulation[23]. The no-slip condition was formulated at the walls, and various flow velocities were subjected at the two inlets.

Fabrication of PDMS microfluidic device

In this research, two types of chips were fabricated: (i) a PDMS-glass and (ii) a PDMS-PDMS layered chip. The standard soft-lithography and replica molding procedure were used to produce the microfluidic devices[24]. The microchip, which was applied to evaluate the mixing performance under a fluorescence microscope, was fabricated using PDMS-glass layering. The microfluidic chip used for determining calcium levels was fabricated based on PDMS-PDMS bonding. With these microfluidic devices, a T-channel with four sets of 10-piece, J-shaped baffles was designed. Each set of baffles was separated by a 1,100-μm long interval. The main channel was 30 mm long, and the width and depth were 500 and 260 μm, respectively.

Evaluation of mixing efficiency

Mixing efficiency of the micromixer was evaluated based on the captured fluorescence images. One syringe

was filled with a solution of 100 μM fluorescein in Milli-Q water (subjected to the inlet), and another syringe was filled with Milli-Q water. The two syringes were injected at various flow rates ranging from 10 μL min^{-1} to 100 μL min^{-1}, using a dual-syringe pump. The mixing of two streams was observed under a fluorescence microscope (BX10, Olympus, Japan) equipped with a CCD camera (DP50, Olympus, Japan). The mixing performance was observed at five positions after passing through each set of J-shaped baffles. The captured images were converted into grey scale before determining the standard deviation of a chosen cross-channel width (5 × 450 pixels area). In this study, the standard deviation (C) of the fluorescent intensity at each area was determined by the equation defined by T. Nguyen *et al.*[10].

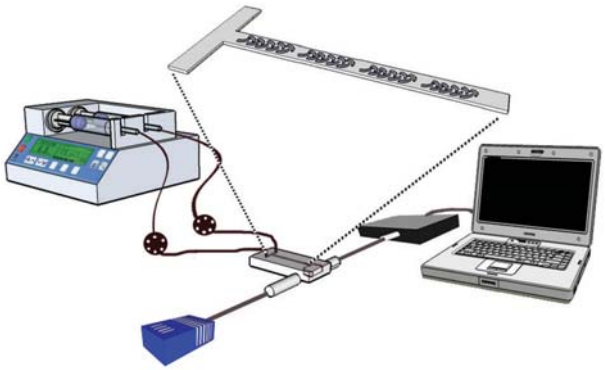

Figure 4. (A) Configuration of the microfluidic system. (B) Schematic layer of the PDMS microchip and detailed view of the passive micromixer with J-shaped baffles.

Microfluidic system set-up

The microfluidic system for calcium level determination included a dual syringe pump (Fusion 200, Chemyx, USA), two six-port injection valves (Upchurch Scientific, USA), a microfluidic micromixer chip, an in-house temperature controller, and a miniature fibre optic spectrometer (USB4000, Ocean Optics Inc., USA), as shown in Figure 4. Unless otherwise stated, all the experiments were performed at 30°C, the carrier buffer used was 25 mM boric acid (pH 8.0), at a constant flow rate of 40 μL min^{-1}. The injection valve was utilised to subject the 2-μL serum samples to the system, while a separate valve was used to load the 25-μL arsenazo III reagent (400 μM). Arsenazo III was selected for determination of serum calcium because it provides high sensitivity, high affinity, and ability to form a stable colour complex with calcium. The intensity of the colour formed is directly proportional to the amount of calcium present in the sample. Before the analysis of calcium, a solution of 1 M HCl was flowed and subsequently washed with the carrier buffer, in order to remove trace contaminations of the calcium ion from the system. When the serum sample and arsenazo III were dispersed together at the inlet of the microchip, a homogeneous mixture was expected downstream after passing each set of 10 J-shaped baffles. The calcium-arsenazo III complexes generated their full colour intensity at the detection zone. After finishing each injection process, the regeneration of the chip was performed by introducing 1 M HCl solution and subsequently washing with a carrier buffer.

Five milliliters of blood samples were drawn from fifteen volunteers with informed consent. Then the clotted blood was centrifuged at 1,500 g for 15 min for separation of serum. Samples from healthy volunteers, including low and high spiked calcium serums (n=15), were assayed via this proposed method. The obtained results were validated with those of a conventional spectrophotometer using a commercial calcium kit from Randox Laboratories. A 650-nm detection wavelength was used according to the manufacturer's instructions for the UV-VIS spectrophotometer (Evolution 600, Thermo Scientific, USA).

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