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Label-free colorimetric sensor for sensitive detection of choline based on DNAzyme-choline oxidase coupling

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Abstract

Changes in choline levels can be associated with diseases such as Alzheimer, Parkinson, Huntington, fatty liver, interstitial lung abnormalities, autism and so on. Therefore, quantitative determination of choline is important in biological and clinical analysis. So far, several methods have been investigated for measuring choline in the body fluids, each of which has disadvantages such as the need for specialist ability, complexity, and high cost. For this purpose, a facile and sensitive colorimetric biosensor based on DNAzyme-choline oxidase coupling used for the determination of choline. In this method, the first, choline oxidase produces H_2O_2 and betaine in the presence of choline and oxygen, then, the DNAzyme converts colorless ABTS into green $ABTS^+$ radicals. Compared to the previous methods, the linear range and the limit of detection of this talented biosensor were 0.1-25 μ*M* and 22 n*M*. Choline measurement using this biosensor has shown satisfactory selectivity and repeatability. Its recovery was 96.95-107.73%, which shows the reliability of biosensor assay in biological samples. Simplicity, low cost, naked eye, high sensitivity, and precision are the benefits of this colorimetric method. Taken to gather, the proposed system can be considered as a great biosensor for measuring choline levels especially in point of care diagnostic.

Keywords: Choline; Colorimetric; DNAzyme

1. Introduction

Choline is produced by synthesis of endogenous but principally from dietary intake [1-3]. Free choline is present in blood and other biological fluids, such as urine, cerebrospinal fluid, and amniotic fluid, within a certain range. Changes in choline levels can be achieved as a result of the consumption of low or high choline content or a disturbance in one of the choline endogenous synthesis pathways [4]. Choline is found in several forms, such as acetylcholine, betaine, and phosphatidylcholine [3]. It is the precursor of the neurotransmitter of acetylcholine that plays an important role in neurodegenerative diseases such as Alzheimer's and Parkinson's disease [5]. Another choline derivative is betaine that changes in its levels in body fluids can be associated with some metabolic abnormalities such as autism in children [6]. In addition, choline in the form of phosphatidylcholine plays an important role in fatty liver disease, interstitial lung abnormalities and Huntington's disease [7-9]. With regards to the influence of choline in human health and pathogenicity, quantitative determination of choline is very important in clinical analysis. There are several methods to measure choline in laboratories. Despite the high sensitivity of conventional chromatography methods, these methods have disadvantages such as time-consuming, the need for experts, the complexity of the methods and high cost. So, new bioanalytical devices based on biosensors replaced the earlier methods [5, 10]. Many biosensors have been designed, such as amperometric enzyme sensor, amperometric aqueous sol–gel biosensor, optical sensor based on fluorescent polymer and chemiluminescent flow sensor. In majority of these biosensors, natural peroxidase has been used as a cognitive element for H_2O_2 catalyst [5].

Colorimetric biosensors are simple, low-cost and cheap compared to other methods [11]. In addition, it can be read out with the naked eyes. Takayama and coworker (1977) used a colorimetric method coupling of choline oxidase and peroxidase to the measurement of choline compounds in serum [12]. In 2004, Campanella and colleagues measured choline compounds in serum, bile and amniotic fluids using a colorimetric method [13]. Also in 2008, Hidaka and coworker used a colorimetric method to determine the amount of sphingomyelin, phosphatidylcholine and lysophosphatidylcholine in serum [14]. In all of these colorimetric methods, natural peroxidase was used for testing. Some disadvantages of

protein enzymes are low stability against hydrolysis, high cost to produce, and complicated preparation and purification [15, 16]. DNAzymes should be superior to many protein-based peroxidase because these catalytic oligonucleotides are self-replicate, more readily to mutate and moderately less expensive to produce [15]. Therefore, it is suggested that the other compounds such as catalytic DNA to be substitute by protein enzyme in colorimetric methods [15, 16]. The DNAzymes are aptamers with catalytic properties that artificially obtained using the SELEX technique [17]. The first time, DNAzyme was synthesized in 1994 by Breaker et al [18]. A kind of DNAzyme that has obtained attention is peroxidase-like DNAzyme [19]. Peroxidase-like DNAzymes catalyze the reaction between hydrogen peroxide and target molecule (ABTS, luminol) which leads to exhibition of oxidized products. This kind of DNAzymes have been employed to design a variety of colorimetric and optical biosensors for the measurement many analytes [20]. Recently, colorimetric biosensors based on DNAzyme have been used to measure cholesterol and sodium in serum samples [21, 22].

In this work the development of a colorimetric biosensor was proposed based on the coupling of the choline oxidase and DNAzyme for the determination of free choline. For this purpose, the laboratory conditions of this biosensor were optimized. Then, the analytical properties of the biosensor were also investigated. In the best of our knowledge it is the first study that report to measure choline with coupling system of choline oxidase and DNAzyme coupled system.

2. Materials and methods

2.1. Materials

The DNA sequences (CatG4-5'- GTGGGTAGGGCGGGTTGG-3') were synthesized and purified by Bioneer company (South Korea). Molar extinction coefficients were determined using UV-Vis absorption spectroscopy. Hemin, ABTS and H_2O_2 were obtained from Sigma. Hemin solution stock (5m*M*) was prepared in DMSO and kept in darkness at -20 °C. Hemin, ABTS and H_2O_2 working solutions were freshly prepared with 10 m*M* Tris-HCl buffer with pH 7.4, containing 0.01% (v/v) Triton X-100, 0.5 µM CatG4, and one of the studied salts

[100 m*M* KCl+100 m*M* NaCl/100 m*M* NaCl+0.8 m*M* $MgCl_2$/1.6 m*M* KCl 0.8 m*M* $MgCl_2$]. Choline oxidase and choline chloride were purchased from Sigma and other reagents were purchased from Merck.

2.2. Preparation of the hemin-G-quadruplexe complex

The DNA solution was dissolved in the 10 m*M* Tris-HCl buffer pH 7.4, containing 0.01% (v/v) Triton X-100, 0.5 µ*M* CatG4, and one of the studied salts [100 m*M* KCl+100 m*M* NaCl/100 m*M* NaCl + 0.8 m*M* $MgCl_2$/ 1.6 m*M* KCl 0.8 m*M* $MgCl_2$]. It was heated at 95 °C for 5 minutes to eliminate any intermolecular interactions and slowly cooled to 25 °C. Then it was incubated at this temperature for 30 minutes to form G-quadruplex DNA. Finally, for the formation of hemin-G-quadruplex complex, The G-quadruplex DNA was incubated with hemin for about 40 minutes in 10 m*M* Tris-HCl buffer [23].

2.3. The method of measuring peroxidase activity

Peroxidase activity analysis of hemin-G-quadruplex complex was performed in the ABTS–H_2O_2 system at room temperature. In this system, before using, the stock solution of ABTS was diluted to 1.4 m*M* with 10 m*M* Tris-HCl buffer with pH 8.0, containing 0.01% (v/v) Triton X-100, 0.5 µM CatG4, and one of the studied salts [100 m*M* KCl+100 m*M* NaCl/100 m*M* NaCl+0.8 m*M* $MgCl_2$/1.6 m*M* KCl 0.8 m*M* $MgCl_2$]. 15 m*M* H_2O_2 was added to the ABTS solution. To this mixture 0.25 µ*M* DNAzyme was quickly added (final concentration). The absorption spectra were recorded every 2 second by an STM UV9000 UV/VIS Spectrophotometer [24].

2.4. Optimization of DNAzyme activity

Considering the importance of DNAzyme performance in the coupled biosensor for the measurement of choline, concentration ratio of G-DNA:hemin, different salt conditions, DNAzyme, ABTS and H_2O_2 concentrations were optimized.

2.4.1. Optimization of concentration ratio of G-DNA:hemin

In this experiment, 1 μ*M* G-DNA and 0.5, 1 and 2 μ*M* hemin were incubated in a 10m*M* Tris-HCl buffer at different concentration ratios (2:1, 1:1 and 1:2), and the formed DNAzyme absorbance was measured at 405 nm.

2.4.2. Optimization of DNAzyme performance under different ionic conditions

DNAzyme was formed under three different ionic conditions, including 100 m*M* KCl+100 m*M* NaCl/100 m*M* NaCl+0.8 m*M* $MgCl_2$ and 1.6 m*M* KCl + 0.8 m*M* MgCl2 in the Tris-HCl buffer with pH 7.4, and the peroxidase activity was measured in the presence of 0.9 m*M* H_2O_2, 1.2 m*M* ABTS and 0.25 μ*M* DNAzyme in the Tris-HCl buffer with pH 8.0 under the ionic conditions listed at 418 nm.

2.4.3. Optimization of ABTS concentration

For the optimization of the ABTS concentration, the radical ABTS absorption changes was measured in the presence of different concentrations of ABTS (2.4, 1.8, 1.2, 0.9, 0.75, 0.6, 0.5, 0.45, 0.4, 0.35, 0.3, 0.25, 0.2, 0.15, 0.1 and 0.05 m*M*), 0.9 m*M* H_2O_2 and 0.25 μ*M* DNAzyme in the Tris-HCl buffer with pH 8.0 at 418 nm.

2.4.4. Optimization of H_2O_2 concentration

In this trial, the radical ABTS absorption changes was measured in the presence of different concentrations of H_2O_2 (1.4, 1.2, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.35, 0.3, 0.25, 0.2, 0.15, 0.1 and 0.05 m*M*), 1.2 m*M* ABTS and 0.25 μ*M* DNAzyme in the Tris-HCl buffer with pH 8.0 at 418 nm.

2.5. Coupling of the choline oxidase-DNAzyme and spectrophotometric determination

Choline content was determined by using a classical spectrophotometric-enzymatic method. This method is based on the two following enzymatic reactions (Scheme 1):

$$\text{Choline chloride} + 2O_2 + H_2O \xrightarrow{Choline\ oxidase} \text{Betaine aldehyde} + 2H_2O_2$$

$$2H_2O_2 + \text{ABTS*} \xrightarrow{DNAzyme} \text{ABTS}^{+} \text{ (green color)} + 4H_2O$$

*2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

In this method, 0.75 U/mL choline oxidase (ChOx) in the 20 m*M* Tris-HCl buffer with pH 8.0 containing 0.01% (v/v) Triton X-100, 1.6 m*M* KCl, 0.8 m*M* $MgCl_2$ and 0.7 m*M* $CaCl_2$ were added into quartz cell containing, 1.2 m*M* ABTS, 35 µ*M* choline chloride and 0.25 µ*M* DNAzyme in 20 m*M* Tris-HCl buffer with pH 8.0, containing 0.01% (v/v) Triton X-100, 1.6 m*M* KCl and 0.8 m*M* $MgCl_2$. All reagents are listed in the final concentration. Finally, absorption changes at 418 nm were measured by using UV-VIS spectrophotometer.

2.6. Optimization of laboratory conditions for choline measurement

2.6.1. Optimization of choline oxidase concentration

Different concentrations of choline oxidase were prepared in 20 m*M* Tris-HCl buffer with pH 8.0, and the optimal concentration of choline oxidase was obtained for this biosensor in the presence of 35 µ*M* choline chloride, 0.25 µ*M* DNAzyme and 1.2 m*M* ABTS at 418 nm.

2.6.2. Optimization of DNAzyme concentration in coupled colorimetric biosensor

Optimization of DNAzyme concentration was performed in the coupled biosensor. For this purpose, various concentrations of DNAzyme (0.5, 0.4, 0.35, 0.25, 0.22, 0.16, 0.13, 0.1, 0.07 and 0.05 µ*M*) in 10 m*M* Tris-HCl buffer with pH 7.4 were prepared and the peroxidase activity was measured in presence the different concentrations of DNAzyme, 35 µ*M* choline, 0.75 U/mL ChOx and 1.2 m*M* ABTS in 20 m*M* Tris-HCl buffer with pH 8.0 at 418nm.

2.6.3. The effect of pH on choline measurement by an enzymatic-colorimetric biosensor

In order to determine the optimal pH for the choline assay the effect of different pHs were measured in the presence of 35 µ*M* of choline chloride, 0.75 U/mL choline oxidase, 0.25 µ*M* of DNAzyme and 1.2 m*M* of ABTS in three different buffers. In this experiment, 20 m*M* sodium acetate (pHs from 4.0-5.5), phosphate (pHs from 6.0-7.0) and Tris-HCl (pHs from 7.5-10.0) buffers were used to reach optimum pH activity.

2.6.4. The effect of temperature on choline measurement by an enzymatic-colorimetric biosensor

To determine the optimum temperature for choline assay the effect of different temperatures (10-60 ºC) were measured in the presence of 35 μ*M* of choline chloride, 0.75 U/mL choline oxidase, 0.25 μ*M* of DNAzyme and 1.2 m*M* of ABTS in Tris-HCl buffer (20 m*M*, pH 8.0).

2.7. Investigation of Repeatability and Precision of the Biosensor

To investigate the repeatability and precision of the biosensor, two test groups (intra-day and inter-day) were designed. Intra-day and inter-day trials were performed under the same conditions, 0.75 U/mL choline oxidase, 0.25 μ*M* DNAzyme, 1.2 m*M* ABTS, and pH 8.0 and the variance of the linear regression equation were calculated for both groups. For intra-day trials, five replicates of the analysis were performed in one day at 2 hours intervals and for inter-day trials, five replicates of the analysis were performed in five consecutive days.

2.8. Investigating the selectivity of enzymatic-colorimetric biosensor for choline measurement

In order to obtain the biosensor specificity, $ABTS^{+}$ absorbance at 418 nm in the presence of 35 μM choline and in the presence similar concentration of different materials (phosphatidylcholine, alanine, arginine, ascorbic acid, asparagine, citric acid, cysteine, galactose, glutamate, glucose, glycine, maltose, phenol, tryptophan, tyrosine, urea, uric acid and glutamine) were measured. In this experiment, the concentration of 0.75 U/mL choline oxidase, 0.25 μM DNAzyme and 1.2 mM ABTS in Tris-HCl buffer (20 mM, pH 8.0) was used.

2.9. Analysis of human serum samples

Human serum samples were obtained by centrifugation of blood for about 5 min with the rotation rate of 3000–4000 rpm. Each sample (10 μL) was added into the reaction mixture (200 μL), which included 0.75 U /mL ChOx, 1.2 m*M* ABTS and 0.25 μ*M* DNAzyme in the final solution. Finally, the recovery was calculated based on the following formula [14, 21]:

$$Recovery = \frac{\text{increased choline concentration in serum}}{a\text{ded choline concentration}} * 100$$

3. Results and discussions

Scheme 1 demonstrates the principle of the DNAzyme-based colorimetric biosensor for detection of choline. Choline is oxidized to betaine and H_2O_2 in the presence of choline oxidase. Then the DNAzyme catalyze of the Redox reaction between H_2O_2 and ABTS. Therefore, the combination of these two reactions could be established a colorimetric biosensor for determination of choline as a result of the color change of $ABTS^+$.

3.1. Demonstration of performance Enzymatic-colorimetric assay

Fig. 1 illustrated the color change and absorption spectrum of $ABTS^+$ under variant conditions. The combination of choline, DNAzyme and ABTS, in the absence of choline oxidase, was colorless (Fig. 1a). Also, the combination of choline oxidase, DNAzyme and ABTS in the absence of choline was colorless (Fig. 1b) but the reaction mixture including choline, choline oxidase, DNAzyme and ABTS changed to green color which represents the good performance of the enzymatic-colorimetric assay (Fig. 1c).

3.2. Optimization of the laboratory conditions for H_2O_2 measurment

The biosensor included two reactions for choline determination. One of these reactions involves measuring the H_2O_2 in the coupled system, during which DNAzyme catalyze the reduction-oxidation reaction of ABTS. Thus any factor involved in this reaction may affect the performance of the biosensor.

3.2.1. Concentration ratio of G-DNA and hemin

The G-DNA and hemin was incubated in order to form DNAzyme in three concentration ratios includeing 2:1, 1:1 and 1:2 G-DNA to hemin and DNAzyme absorbance investigated at 405 nm. The results showed the G-DNA-hemin DNAzyme at a concentration ratio of 2:1 at 405 nm has a lower absorption peak (Fig. 2a), which may be due to insufficient hemin concentration compared to the G-quadruplex. Also G-DNA-hemin complex at a concentration ratio of 1:2, did not show an increase in adsorption compared to 1:1

concentration ratio. This can be due to a sufficient concentration of hemin at a 1:1 concentration ratio. Hence, the G-DNA-hemin complex at a concentration ratio of 1:1, was used to carry out more experiments. Kong and coworker used the G-DNA-hemin complex at a concentration ratio of 1:2 and 1:1 to measure peroxidase activity and to study of G-DNA structure, respectively [23]. Shahbazi and coworker used the G-DNA-hemin complex at a concentration ratio of 1:1 in the study of DNAzyme structure and performance [25].

3.2.2. Measurement peroxidase activity in the presence of different ions

Metal ions play a crucial role in the formation of G-quadroplex. Chen and coworkers showed the presence of potassium ion on the formation of a stable DNAzyme and relationship between G-quadroplex topology and oligonucleotide enzyme activity [26]. Therefore, peroxidase activity of CatG4 DNAzyme was investigated in the presence of different ion conditions, and it was shown that the best of peroxidase activity obtained in the presence of 1.6 m*M* KCl and 0.8 m*M* $MgCl_2$ (Fig. 2b). This difference in the DNAzyme peroxidase activity is due to differences in the formation of the G-quadroplex structure and its ability to connect to hemin [16]. Kong and coworkers investigated the effect of 100 m*M* NaCl/100 m*M* KCl/1.6 m*M* KCl + 0.8 m*M* $MgCl_2$ on the catalytic activity of CatG4 DNAzyme and showed that optimal activity of DNAzyme was in the presence of 1.6 m*M* KCl+0.8 m*M* $MgCl_2$ [23]. Kosman and coworkers investigated the effects of K^+, NH_4^+ and Sr^{2+} on the catalytic activity of PS2M DNAzyme and obtained optimal activity of DNAzyme in the presence of potassium ion [20].

3.2.3. Optimization of the ABTS concentration

The oxidation of ABTS by hydrogen peroxide in the presence of DNAzyme leads to radical $ABTS^+$ product with rapid kinetic reaction that makes it suitable for instrumental analysis. So, optimization of the ABTS concentration is important for choline assay. The concentration of ABTS was investigated in over the range of 0.05 to 2.4 m*M*, as shown in the Figure 3a, the absorbance at 418 nm increased with an increase in the concentration of ABTS in the range of 0.05 to 1.2 μ*M*, but it maintained on the plateau region at higher concentrations. Therbey 1.2 m*M* ABTS was selected as optimal concentration for the detection of choline. Li and coworkers used a concentration of 5.9 m*M* ABTS to the detection of thrombin [27]. Sun and

coworkers used 5 m*M* ABTS for sodium assay in serum sample [22]. Zhou and coworkers used a concentration of 3.2 m*M* ABTS to measure Ag^+ and cysteine [28].

3.2.4. Optimization of the H_2O_2 concentration

Considering the great effect of H_2O_2 on the catalytic activity of DNAzyme, the effect of H_2O_2 concentration in over the range of 0.05 to 1.4 m*M* was studied and, as shown in the Figure 3b, absorbance was increased in the range of 0.05 to 0.9 m*M*, but it kept on the plateau region at higher concentrations. Therefore, 0.9 m*M* H_2O_2 was used as optimal concentration for the detection of choline. Zhou and his coworkers used the concentration of 2.2 m*M* H_2O_2 for detection of Ag^+ and cysteine [28]. Kong et al. measured peroxidase activity of CatG4 DNAzyme at a concentration of 1.9 m*M* H_2O_2 [23]. In these cases, a higher concentration of H_2O_2 has been used comparing the present study.

3.3. Optimization of the laboratory conditions for choline measurement

The choline oxidase catalyzes the reduction-oxidation reaction of choline. Therefore, this reaction plays a very important role in the measurement of choline in the coupled biosensor and optimization the factors affecting on this reaction improves the colorimetric biosensor performance.

3.3.1. Optimization of the choline oxidase concentration

Choline oxidase plays an important role in the production of H_2O_2 from choline, thus its concentration optimization is very important. So, the concentration of choline oxidase in the range of 0.1 to 1.2 U/mL was investigated. As shown in the Figure 4a, in the range of 0.1 to 0.75 U/mL choline oxidase the absorbance increased whereas at higher concentrations, the absorbance curve maintained on the plateau region. Thereby 0.75 U/mL choline oxidase was used for the detection of choline. It is lower than the results of Takayama and coworkers [12] and Companla et al. [29] which used 1.0 and 10 U/mL concentrations of choline oxidase to the measurement of serum choline in the colorimetric method, respectively. Because of high

cost of choline oxidase production, the lower using of choline oxidase in biosensing system indicating that the excellent system optimization, especially in large scale using.

3.3.2. Optimization of the DNAzyme concentration

DNAzyme catalyze the reaction of oxidation-reduction in the presence of hydrogen peroxide and ABTS, so it is important as a cognitive element in this biosensor. For this reason the concentration of DNAzyme in the range of 0.05 to 0.5 μ*M* was prepared in a 10 m*M* Tris-Hcl buffer (pH 8.0) and the effect of DNAzyme concentration on choline measurement was studied. As shown in the Figure 4b, the absorbance at 418 nm has increased with an increase in the concentration of DNAzyme in the range of 0.05 to 0.25 μ*M*, but has not been increased at higher concentrations. Consequently, 0.25 μ*M* DNAzyme was used for further experiments. It is lower than the results of Wang et al. [30] and Zhou et al. [28] which used 0.35 μ*M* PW17 DNAzyme and 0.3 μ*M* CatG4DNAzyme in their studies, respectively. It is indicated that, this biosensor-based DNAzyme is inexpensive for biosensing of analyte.

3.3.3. Optimization of the pH value

The pH of a solution could have several effects on the structure and activity of enzymes. Therefore, the effect of pH has been investigated in the range of 4.0 to 10.0 and, as shown in the Figure 4c, the amount of absorbance at 418 nm increased in the range of pH from 4.0 to 8.0, while the absorbance decreased at higher pH values. So, pH 8.0 was considered as optimal temperature for the biosensing optimization. Since, this pH is close to the physiological range, it make it a valuable biosensor to measure choline in the biological samples. The same results was also obtained by Li and coworkers for detection of cholesterol in the serum at pH 8.0. Sun and coworkers used also a DNAzyme based colorimetric assay to measure sodium in the serum at pH 6.8 [22]. But, Li and coworkers obtained the highest luminescence intensity at optimal pH of 3.0 in a luminescent biosensor for choline measurement [31].

3.3.4. Optimization of the temperature

Temperature is one of the important factors affecting the structure and activity of DNAzyme. So, the temperature plays a significant role in the performance of the biosensor for choline detection. Hence, the reaction temperature was investigated in the temperature range of 10 to 60 ºC. As shown in the Figure 4d, the absorbance at 418 nm increased in the range of 10 to 25 ºC, while the absorbance is reduced at higher temperatures of 25 ºC. Thereby, 25 °C was selected for the further study. The same results was also obtained by Li and coworkers [31], which showed that 25 °C was an optimal temperature for cholesterol measurements in a colorimetric method based on DNAzyme. Song and coworkers [32], Li and coworkers [5] reported that 30 and 35 °C were the optimal temperature in the performance of amprometric and fluorescent biosensors, respectively. Our results indicated that this colorimetric biosensor is a talented choline biosensing especially in point of care diagnostic.

3.4. Repeatability of enzymatic-colorimetric biosensor

The ability of a biosensor to generate similar responses for a set of experiments with the same conditions is called repeatability [33]. The repeatability of the enzymatic-colorimetric biosensor was investigated and the RSD value was calculated for the linear regression equation slopes and intercepts below 3.68 % for intra-day and 5.7 % for inter-day, respectively (Table 1). These results indicate good repeatability of this biosensor for choline assay. Li et al. [21] also reported the same colorimetric biosensing system for determination of cholesterol. Their results showed the relative standard deviation for the slops and intercepts of the linear regression equation were 5.4% and 7.2%, respectively.

3.5. Calibration plot for choline (Linearity and LOD)

The relationship between choline concentration and absorbance studied at 418 nm in the range of 0.0 to 80 μ*M*. As shown in the Figure 5, the choline concentration is proportional to the absorbance in the range of 0.1 to 25 μ*M* that suggests this biosensing ststem has an acceptable linearity for choline measurement in body fluids including serum and plasma. In Table 2, this parameter is compared with the linearity of some existing biosensors for choline measurement. Also the linear regression equation obtained as y=0.0225x+0.1178 (R^2=0.9967). Another important parameter that examined in choline biosensor was the limit

of detection that obtained 22 n*M* (3× SD/Slop) [21, 34]. The limit of detection for this biosensor indicates the high sensitivity of this method compared to many electrochemical and amperometric biosensors (Table 2). Up to now, there isn't any colorimetric biosensing system for choline determination based on choline oxidase-DNAzyme coupling. As shown in Table 2, our results indicated that, the Linearity and LOD of this biosensor is better than some of the electrochemical, fluorescent and amprometric biosensors such as the biosensors reported by Tiagarajan et al. [1], Li et al. [5] and Mazurenko et al. [35] respectively. It is mentioned that, the linear region of these electrochemical (0.3-5.1 mM) and amprometric (200-600 μM) biosensors is not suitable for measuring the micromolar concentrations of choline in biological fluids such as serum and plasma (7-13.3 μM) [4]. Wu et al. [36] also reported an electrochemiluminescence biosensor to measure choline. Although, the linearity and the LOD of this biosensor is better than the choline oxidase-DNAzyme coupling, but this biosensor constructed with high consumption of reagents, high costs, more complicated preparation and environmental pollution. However, the present biosensor with excellent properties is cheaper, simpler and it can be used in the point of care diagnostic without any instrument.

3.6. The selectivity of enzymatic-colorimetric coupling assay

The selectivity for this colorimetric biosensing system was also performed. Selectivity, an important feature of the biosensor, is the ability of a biological receptor to detect a particular analyte in a sample containing other compounds and additives [37]. The selection of interfering substances including phosphatidylcholine, alanine, arginine, ascorbic acid, asparagine, citric acid, cysteine, galactose, glutamate, glucose, glycine, maltose, phenol, tryptophan, tyrosine, urea, uric acid and glutamine is based on their presence in biological fluids including serum. As demonstrated in the Figure 6, there is a significant difference between the detection of choline (absorbance of 0.7 for a concentration of 35μ*M*) and other interfering substances (absorbance below 0.05 for a concentration of 35 μ*M*), which indicates this biosensor has satisfactory selectivity. It is mentioned that Kucherenko et al. used a semi-permeable membrane to decrease the interference of different materials in amperometric biosensor and they succeeded to limit the interference of uric acid, dopamine, ascorbic acid

and cysteine [10]. But the present biosensor has great selectivity to choline without any additive compounds, which indicated that it is cheap and useful especially in point of care diagnostic.

3.7. Determination of choline in human serum

In order to evaluate the practicability of the choline assay in real samples, choline concentration was measured in serum of different individuals. As shown in Table 3, choline concentration in human serums measured in the range of 9.97 to 11.6 μ*M*, which were in complete agreement with previous reports [4]. The recovery were determined in the range of 96.95% to 103.7%, which indicates the reliability of these results.

Conclusions

Regarding the role of choline in the health and pathogens of the human body, in the present study, we designed an enzymatic-colorimetric biosensor based on choline oxidase-DNAzyme coupling. This biosensor is simpler and cheaper than amprometric and electrochemical methods and has shown a great sensitivity (22 n*M*) with a wide linearity of 0.1-25 μ*M*, compare to the previous studies. In addition, this biosensor indicated satisfactory repeatability and selectivity for choline measurement. Eventually, this biosensor was used to measure free choline in human serum samples, and the recovery calculated in the range of 96.95-103.7%, indicating that the results obtained from this biosensor are reliable. Taken to gather, these unique properties indicated that it is a talented biosensor for choline detection in biological samples, especially in point of care diagnostic.

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Figure legend:

Figure1. Color change and absorption spectrum of $ABTS^+$ under variant conditions.

Figure2. Effect of various concentrations of G-DNA/hemin (a) and different ionic conditions (b) on the absorbance change of the solution. The experiments were carried out in 10 mM Tris-HCl buffer solution containing 0.01 % triton X-100. a) 0.8 m*M* $MgCl_2$ + 1.6 m*M* KCl, 1.2 mM ABTS, 0.9 m*M* H_2O_2, pH=8.0, 25 °C; b) 0.25 µ*M* DNAzyme (DZ), 1.2 m*M* ABTS, 0.9 m*M* H_2O_2 pH=8.0, 25 °C.

Figure3. Effect of various concentrations of H_2O_2 (a) and ABTS (b) on the absorbance change of the solution. The experiments were carried out in 10 m*M* Tris-HCl buffer solution containing 0.01 % triton X-100, 0.8 m*M* $MgCl_2$ and 1.6 m*M* KCl. a) 0.25 µ*M* DNAzyme, 0.9 m*M* H_2O_2, pH= 8.0, 25 °C; b) 0.25 µ*M* DNAzyme, 1.2 m*M* ABTS, pH= 8.0, 25 °C.

Figure4. Effect of the concentrations of choline oxidase (a), DNAzyme (b), pH (c) and temperature (d) on the absorbance change of the solution. The experiments were carried out in 20 m*M* Tris-HCl buffer solution containing 0.01 % triton X-100, 0.8 m*M* $MgCl_2$, 1.6 m*M* KCl and 0.7 m*M* $CaCl_2$. a) 35 µ*M* choline, 0.25 µ*M* DNAzyme, 1.2 m*M* ABTS, pH= 8.0, 25 °C; b) 35 µ*M* choline, 0.75 m*M* choline oxidase, 1.2 m*M* ABTS, pH= 8.0, 25 °C; c) 35 µ*M* choline, 0.75 m*M* choline oxidase, 0.25 µ*M* DNAzyme, 1.2 m*M* ABTS, 25 °C; d) 35 µ*M* choline, 0.75 m*M* choline oxidase, 0.25 µ*M* DNAzyme, 1.2 m*M* ABTS, pH= 8.0.

Figure5. The relationship between choline concentration and absorbance at 418 nm. All measurements were carried out in 20 m*M* Tris–HCl buffer solution with pH 8.0 containing 0.8 m*M* $MgCl_2$, 1.6 m*M* KCl, 0.7 m*M* $CaCl_2$, 0.25 µ*M* DNAzyme, 0.75 U/mL ChOx, 1.2 m*M* $ABTS^+$ and 25 °C.

Figure6. Selectivity measurement in the presence of interfering substances. All measurements were carried out in 20 m*M* Tris–HCl buffer solution with pH 8.0 containing 0.8 m*M* $MgCl_2$, 1.6 m*M* KCl, 0.7 m*M* $CaCl_2$, 0.25 µ*M* DNAzyme, 0.75 U/mL ChOx, 1.2 m*M* $ABTS^+$ and 25 °C.

Table 1

RSD based on the slope and intercept of the linear regression equations

Assay	Slop	RSD (%, n=5)	Intercept	RSD (%, n=5)
Intra-day	0.0237	2.53	0.1183	3.45
Inter-day	0.0245	3.68	0.1174	5.70

Table 2

Comparison the current work with other reports.

Sensing element	Transducer used	Linear range	Limit of detection	References
GCE/Chit/ZnO/ChOx electrode	Electerochemical	0.3-5.1 m*M*	0.58 m*M*	1
Fluorescent plymer/ChOx-HRP	Fluorescent	0.1-20 µ*M*	50 n*M*	5
AuSPE-SiO2-ChOx	Amperometric	200-600µ*M*	6 µ*M*	39
ChOx/titanate nanotubes/chitosan	Electerochemical	3n*M*-1.12 m*M*	1 n*M*	40
ChOx/DNAzyme	Colorimetric	0.1-25 µ*M*	22 n*M*	Present work

Table 3

Free choline measurement in serum samples

Sample	Free choline in serum (μ*M*)	Choline added (μ*M*)	Increased choline (μ*M*)	Recovery (%)	RSD (%, n=5)
Serum1	10.74	10	10.25	102.5	0.10
Serum2	10.92	10	10.25	102.5	0.08
Serum3	9.97	10	9.82	98.2	0.10
Serum4	10.15	10	10.16	101.6	0.07
Serum5	9.85	10	9.69	96.9	0.12
Serum6	11.60	10	10.37	103.7	0.09

Highlights

- This is the first report of colorimetric detection of choline based on DNAzyme-choline oxidase coupling
- The linear range of this biosensor was 0.1-25 μM.
- The limit of detection of this biosensor was 22 nM.
- The recovery from choline assay in human serum was 96.9-107.7%.
- Simplicity, naked eye, high sensitivity are the benefits of this colorimetric method.

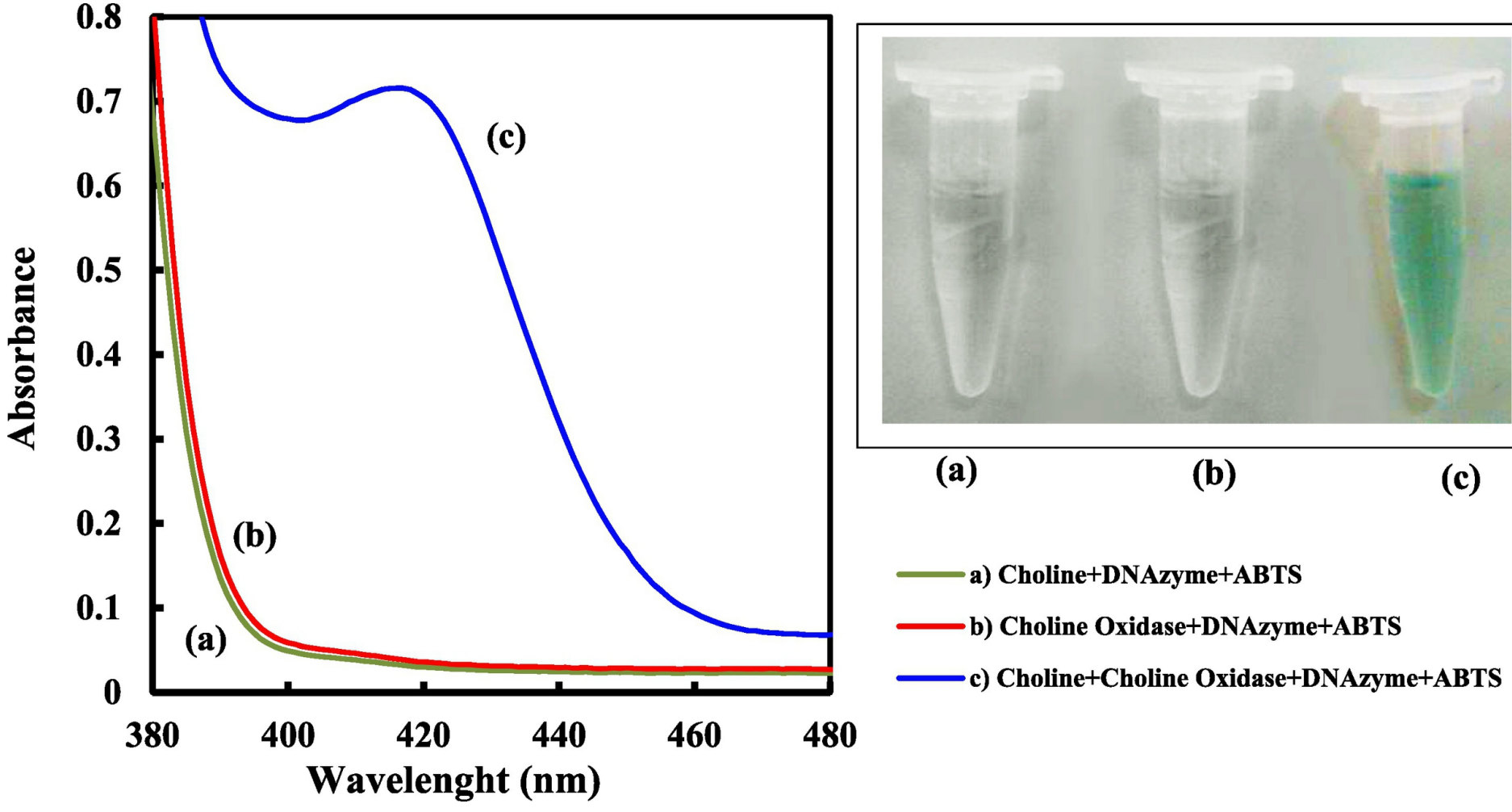
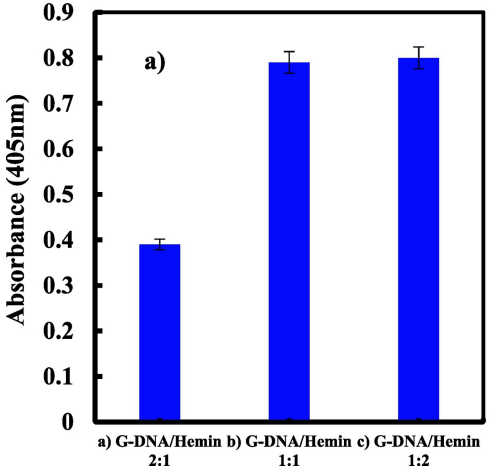

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbance
380
400
420
440
460
480
Wavelenght (nm)
(a)
(b)
(c)
(a)
(b)
(c)
a) Choline+DNAzyme+ABTS
b) Choline Oxidase+DNAzyme+ABTS
c) Choline+Choline Oxidase+DNAzyme+ABTS

Figure 1


a)
Absorbance (405nm)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
a) G-DNA/Hemin 2:1
b) G-DNA/Hemin 1:1
c) G-DNA/Hemin 1:2

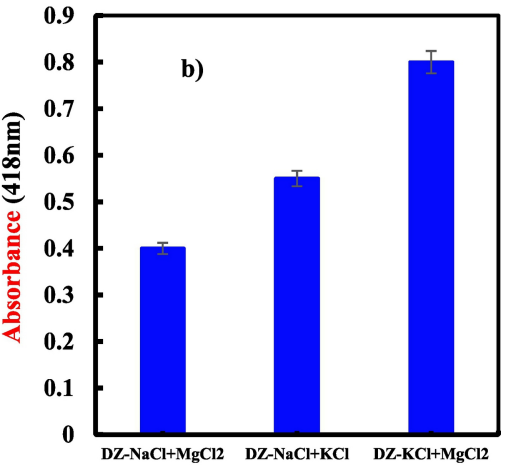

b)
Absorbance (418nm)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
DZ-NaCl+MgCl2
DZ-NaCl+KCl
DZ-KCl+MgCl2

Figure 2

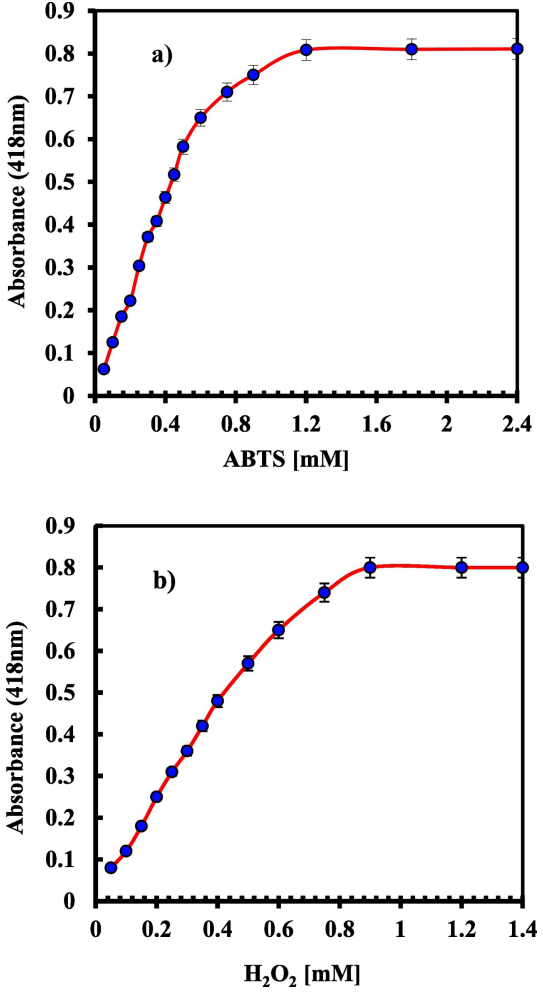

a)
Absorbance (418nm)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.4
0.8
1.2
1.6
2
2.4
ABTS [mM]
b)
Absorbance (418nm)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.2
0.4
0.6
0.8
1
1.2
1.4
H2O2 [mM]

Figure 3

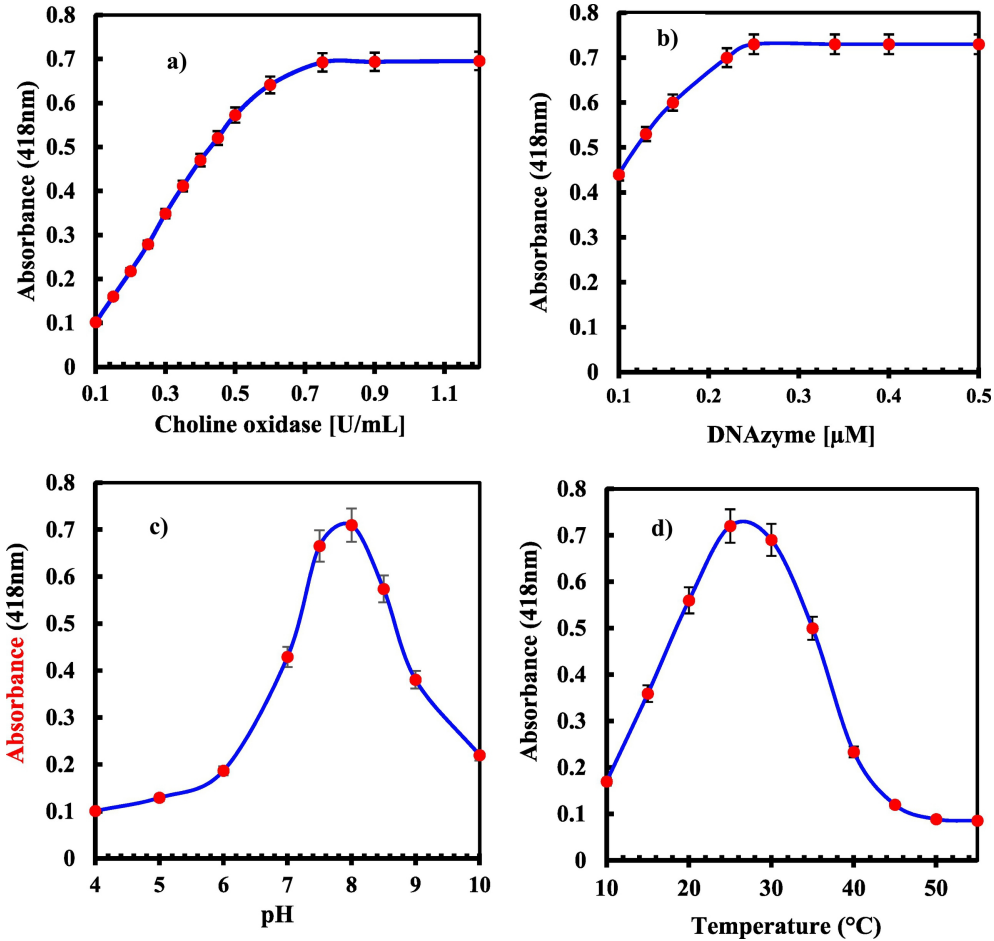

a)
Absorbance (418nm)
Choline oxidase [U/mL]
b)
Absorbance (418nm)
DNAzyme [µM]
c)
Absorbance (418nm)
pH
d)
Absorbance (418nm)
Temperature (°C)

Figure 4

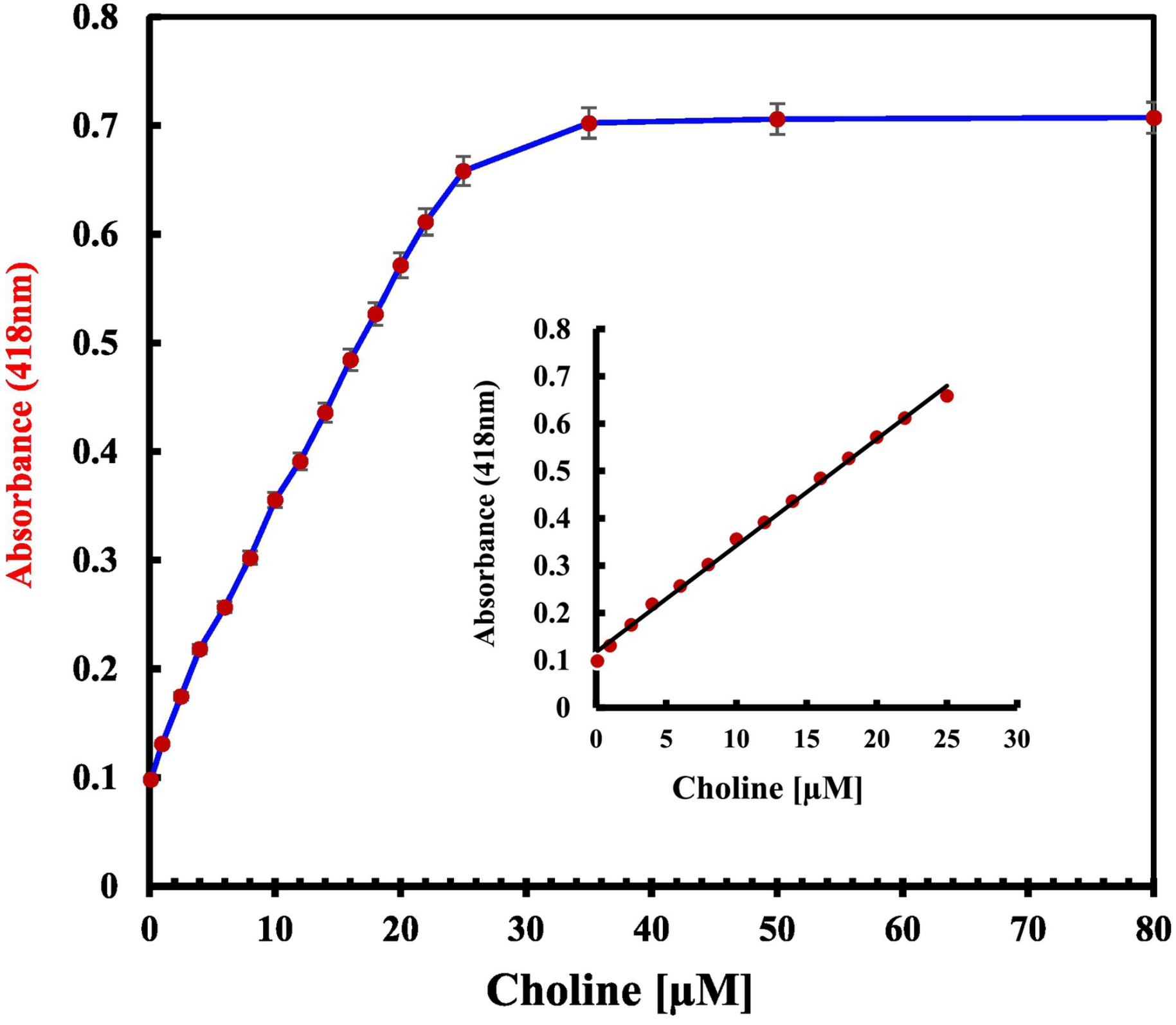

Absorbance (418nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
60
70
80
Choline [μM]
Absorbance (418nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
30
Choline [μM]

Figure 5

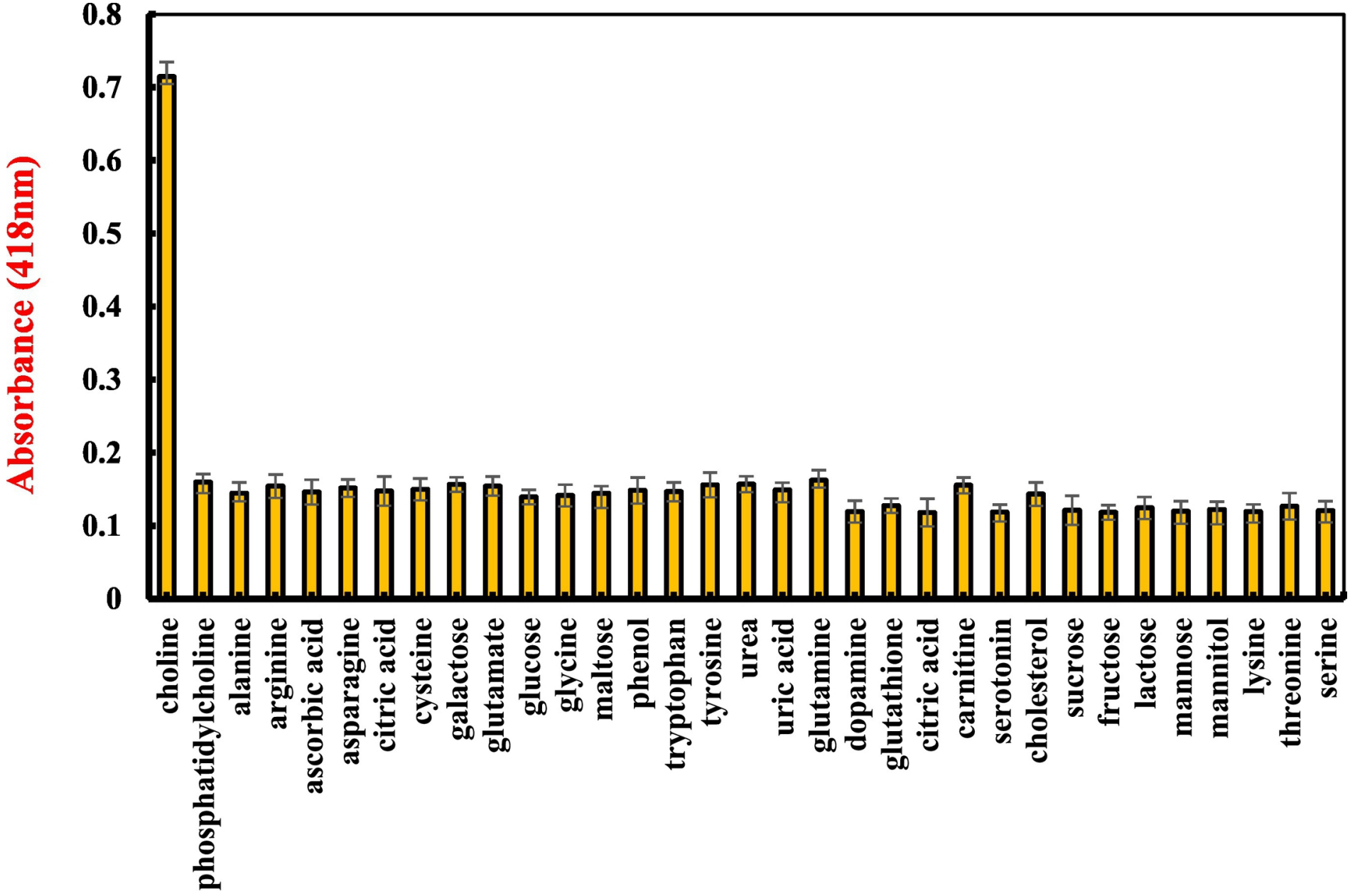

Absorbance (418nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
choline
phosphatidylcholine
alanine
arginine
ascorbic acid
asparagine
citric acid
cysteine
galactose
glutamate
glucose
glycine
maltose
phenol
tryptophan
tyrosine
urea
uric acid
glutamine
dopamine
glutathione
citric acid
carnitine
serotonin
cholesterol
sucrose
fructose
lactose
mannose
mannitol
lysine
threonine
serine

Figure 6