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Rapid high-throughput analysis of ochratoxin A by the self-assembly of DNAzyme–aptamer conjugates in wine

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ABSTRACT

We report a new label-free colorimetric aptasensor based on DNAzyme–aptamer conjugate for rapid and high-throughput detection of Ochratoxin A (OTA, a possible human carcinogen, group 2B) in wine. Two oligonucleotides were designed for this detection. One is **N1** for biorecognition, which includes two adjacent sequences: the OTA-specific aptamer sequence and the horseradish peroxidase (HRP)-mimicking DNAzyme sequence. The other is a blocking DNA (**B2**), which is partially complementary to a part of the OTA aptamer and partially complementary to a part of the DNAzyme. The existence of OTA reduces the hybridization between **N1** and **B2**. Thus, the activity of the non-hybridized DNAzyme is linearly correlated with the concentration of OTA up to 30 nM with a limit of detection of 4 nM (3σ). Meanwhile, a double liquid–liquid extraction (LLE) method is accordingly developed to purify OTA from wine. Compared with the existing HPLC-FD or immunoassay methods, the proposed strategy presents the most appropriate balance between accuracy and facility, resulting in a considerable improvement of real-time quality control, and thereby, preventing chronic poisoning caused by OTA contained red wine.

1. Introduction

OTA is the most common naturally occurring mycotoxin, which is a secondary metabolite of fungi strands like *Aspergillus ochraceus*, *Aspergillus carbonarius* and *Penicillium verrucosum* (FAO/WHO 2001). OTA is nephrotoxic and is suspected of being the main etiological agent responsible for human Balkan endemic nephropathy (BEN) and associated urinary tract tumors. OTA also exhibits immunosuppressive, teratogenic and carcinogenic properties [1]. The International Agency for Research on Cancer has classified OTA as a possible human carcinogen (group 2B). Improperly stored (for example high temperature, high level of humidity, and other storage conditions) food products and food commodities, such as cereals, spices, dried fruits, coffee beans, beer and wine are likely to be contaminated with this toxin [2]. In the European diet, grape products, especially red wine, have been identified as the second major source (corresponding to 13%) of human exposure to OTA, following cereals [3]. As a consequence, maximum tolerated OTA levels have been established by several countries in individual regulations. For OTA in wine and grape-based beverages, the European Commission (EC no. 123/2005) has set the maximum levels at 2 μg/kg (equivalent to 5 nM).

Together with preventive measures, methods for OTA screening in contaminated raw materials are of great importance to avoid the risk of toxin consumption. Due to the natural fluorescence of OTA, chromatographic techniques have been usually taken as reference methods (high performance liquid chromatography coupled with fluorescence detection, HPLC-FD) [2,4,5]. Despite their accuracy and low limit of detection, these techniques require sophisticated equipments and trained personnel. Biosensors and bioassays have been widely developed for OTA detection. The sensitive biological elements include antibody, peptide, molecular imprinted polymer (MIP), etc. Immunoassays based on antigen–antibody reactions such as enzyme linked immuno sorbent assays (ELISA) [6,7], flow-through enzyme immunoassay [8] and electrochemical immunosensors [9,10] are popular alternatives for OTA detection in wine. Although the limits of detection of these assays are quite low, a stable supply of antibodies are often required, which is a laborious, expensive, and time-consuming procedure with limited reusability. MIP replaces the biorecognition element of immunoassays by a less expensive and more stable biomimetic counterpart. MIP is highly

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selective in polar and protic media and OTA is bound with significantly high affinity. The template necessary during the process of MIP preparation is OTA, but this can be harmful to the operator [11].

Except being the blueprint of life, nucleic acids have the ability to perform other interesting functions, including ligand binding and catalysis. These kinds of nucleic acids are known as functional nucleic acids (FNAs) [12]. In this paper, two kinds of FNAs are used. One is an aptamer, which is a single stranded oligonucleotides selected *in vitro* by the systematic evolution of ligands by exponential enrichment (SELEX) process from random-sequence nucleic acids libraries [13,14]. Due to inherent selectivity and affinity, aptamers can rival to some degree with antibodies as recognition elements [15]. Compared with ochratoxin B (OTB), which is the dechlorinated analog of OTA, OTA aptamer exhibits impressive binding specificity to OTA (approximately 100-fold higher affinity than OTB) [16,17]. A couple of aptamer assays have been developed as a promising tool for OTA detection, such as enzyme-linked aptamer assays [18], electrochemical method [19,20] and structure-switch signaling aptamer [21]. The other FNA is a HRP-mimicking DNAzyme, which can catalyze a chemical reaction to generate a colorimetric signal. G-rich oligonucleotides can form a four-stranded structure known as G-quadruplex. Hemin as a ligand can specifically bind to several parallel G-quadruplexes with high affinity. These hemin-G-quadruplex complexes behave as a HRP-mimicking DNAzyme that may display a highly enhanced catalytic activity compared with hemin itself [12,22]. For label-free assays, these DNAzymes are an alternative to the use of enzymes in aptamer-based biosensors [23,24].

To protect the consumers from the harmful effects of OTA, some new analytical methods are highly desired, which is expected to be a convenient, economical, time-efficient, user-friendly and sensitive measurement without complex instruments. Here we have developed a label-free colorimetric aptasensor to detect OTA.

Rapid high-throughput analysis of OTA in wine depends not only on rapid detection process but also on rapid sample clean-up process. Immunoaffinity column (IAC) is one of the major clean-up techniques and also the approach proposed by the European Committee (EC no.401/2006) for standardization of the clean-up of OTA in wine [25]. Although IACs become increasingly popular, they are expensive, not always reusable and have a limited storage life. IACs are based on specific interaction of antigen and antibody, but the process to produce antibody is laborious. For these columns, acidic solvents cannot be applied because the antibody might be damaged [2]. Except IACs, there are various extraction methodologies for sample preparation and clean-up, such as solid phase extraction (SPE), molecularly imprinted polymer SPE (MIP-SPE), immuno-ultrafiltration [2] and aptamer solid phase extraction affinity columns [26–28], etc. Unfortunately, MIP-SPE requires a template and immuno-ultrafiltration uses bio-recognition elements (antibody), and SPE is more simple but unselective. Compared with IACs, aptamer solid phase extraction affinity columns are reusable and relatively cheap. To sum up, at least one column needs to be prepared for all these methods, leading to a costly, labor-intensive and time-consuming process. For matching our assay, we have developed a double liquid–liquid extraction (LLE) method to purify OTA from wine. After purification, the colorimetric bioassay based on OTA aptamer and DNAzyme was carried out to detect the concentration of OTA in wine.

2. Experimental section

2.1. Reagents

Sodium chloride (NaCl), tris(hydroxymethyl)aminomethane ($(HOCH_2)_3CNH_2$) (Tris), and magnesium chloride ($MgCl_2$) were purchased from Sigma (France). All chemicals were at least of analytical grade. OTA (from *A. ochraceus*), 3, 3′, 5, 5′-tetramethylbenzidine (TMB) liquid substrate and components of buffers were purchased from Sigma (France). OTB (from *A. ochraceus*) was purchased from Santa Cruz Biotechnology (Germany). Warfarin was obtained from Sigma-Aldrich (St. Louis, MO, USA). The ssDNA oligonucleotides were synthesized by Eurogentec (Belgium). The sequence of the new DNA (**N1**) was 5′-TGGGT AGGGC GGGTT GGGAA *AGATC GGGTG TGGGT GGCGT AAAGG GAGCA TCGGA CA*-3′, the sequence with underline could form DNAzyme [24] and the sequence with italic is OTA aptamer [16,17]. The sequence of the blocker (**B2**) was 5′-*CACAC CCGA*A AAATC CCAAC CC-3′. The DNA stock solution was obtained by dissolving the oligonucleotide in 10 mM Tris–HCl buffer containing 120 mM NaCl (pH 8.4) and was stored at 4 °C before use. The water used throughout all experiments was purified by a Milli-Q system (Millipore, Bedford, MA, USA). 96-Well Polysorp microtiter plates were obtained from Nunc (Roskilde, Denmark).

2.2. Instruments

Colorimetric measurements were performed with a Labsystems Multiskan EX microtiter plate reader (Thermo Life Sciences, Cergy-Pontoise, France). Fluorescence measurements were performed with a fluorescence spectrometer LS55 (Perkin-Elmer, MA, USA). UV/VIS studies were carried out using a Varian Cary 50 UV/VIS spectrophotometer (Varian Com. US).

2.3. Detection of OTA using colorimetric biosensing method

In a typical experiment, 100 μL of solution containing 50 nM of **N1** and different concentrations of analyte (OTA) in 10 mM Tris–HCl buffer (pH 8.4) with 120 mM NaCl, 2.5 mM $MgCl_2$, 5 mM KCl and 100 nM hemin were added into 96-well microplate and incubated for 10 min. After that, 5 μL of 1 μM **B2** was added, mixed and incubated for 10 min, and then 20 μL of TMB (including H_2O_2) was added and mixed. Subsequently, absorbance was measured in a kinetics mode at wavelength of 620 nm for 30 min. Each point was the mean of three experiments. All assays were performed at room temperature.

For checking the assay specificity, OTB, the dechlorinated analog of OTA, and warfarin were used in the same protocol as described above.

2.4. Preparation of wine samples with double LLE

The red wine samples used in this study were all purchased from a supermarket in China. The wine was analyzed by classical method using HPLC and fluorescence detection to be sure that it was not contaminated with OTA. The red wine was spiked with the stock solution of OTA to obtain a final concentration of 312.5 nM OTA. From this spiked sample, several dilutions from 2.4 nM to 156 nM OTA were prepared with non-spiked red wine. The red wine samples were mixed with the same volume of toluene. After complete phase separation, a given amount from each top organic phase was taken out for the next step. In this step, the top organic phases were mixed with the same volume of 10 mM Tris–HCl buffer containing 120 mM NaCl (pH 8.4). After complete phase separation, a given amount of each bottom aqueous phase was taken out, adjusted the pH to 3 by the addition of hydrochloric acid, and then mixed with the same volume of chloroform. After complete phase separation, a given amount of each bottom organic phase was taken out, mixed with the same volume of 10 mM Tris–HCl buffer containing 120 mM NaCl (pH 8.4). After complete phase separation, a given amount of each top aqueous phase was taken out and used for detection.

2.5. Colorimetric biosensing method for OTA detection in wine

The detection solution was composed of 500 nM **N1**, 25 mM $MgCl_2$, 50 mM KCl and 1 μM hemin. 90 μL of wine samples processed with double LLE were dispensed into the 96-well polysorp microtiter plate, and then 10 μL detection solution was added. After mixing, the microtiter plates were left to react for 10 min. After that, 5 μL of 1 μM **B2** was added, mixed and incubated for 10 min, and then 20 μL of TMB (including H_2O_2) was added and mixed. Subsequently, absorbance was measured in a kinetics mode at 620 nm for 30 min. Each point was the mean of three experiments. All assays were performed at room temperature.

3. Results and discussion

3.1. Principle of analysis of OTA

The principle of the bioassay is depicted in Fig. 1. In this assay, two oligonucleotides were designed: **N1** which includes OTA aptamer and DNAzyme, as shown in Fig. 1; a blocking DNA (**B2**) which is partially complementary to a part of the OTA aptamer and to a part of the DNAzyme, resulting in an inactive DNAzyme. Upon the addition of OTA, the conformation of OTA aptamer changes from random coil structure to antiparallel G-quadruplex structure (a rigid structure) [29]. Upon the addition of K^+ and hemin [22], the selected G-rich sequence forms a parallel hemin–G-quadruplex complex which behaves as a kind of peroxidase-like DNAzyme and displays a highly enhanced catalytic activity compared to hemin itself. The structure of OTA aptamer is antiparallel, so it does not contribute to the activity of HRP-mimicking DNAzyme [22]. The degree of completeness of the parallel G-quadruplex structure will influence the catalytic efficiency of DNAzyme. When OTA, K^+ and hemin exist at same time, the bases of **N1** compose two G-quadruplex complexes, these rigid structures cause less exposed bases which affects the hybridization kinetics [30]. Hence, when **B2** is added, the possible hybridization of **N1** with **B2** is less efficient. Without OTA, **B2** would easily hybridize with the random coil structure of OTA aptamer. Further, **B2** would hybridize with the selected G-rich sequence [31]. The free, non-hybridized DNAzyme is active and its activity is monitored spectrophotometrically via the H_2O_2 mediated-oxidation of TMB. Therefore, the analytical readout of such activity can be correlated with the concentration of OTA.

The chosen G-rich sequence is known for mimicking an HRP-like enzyme [22]. Hence, the Henri–Michaelis–Menten equation is applied for describing its activity (1). At very high concentrations of substrate, the enzymatic reaction rate is essentially independent of the substrate concentration. This is the region of *zero-order kinetics*. In this case, the slope of the kinetics measurements (ν_{max}) is dependent on the activity of peroxidase-like DNAzyme, meaningly on the **N1** concentration. In order to keep the condition $[S] \gg K_m$, we have used the concentrated commercial TMB liquid substrate in a reduced volume (20 μL) to assure also a low dilution of **N1** concentration. The slope of the time-dependent changes in absorbance (ν) is correlated with the DNAzyme activity. Compared with the absolute difference of two absorbance values, the slope of the entire kinetic profile is preferred as it is subjected to less random errors. This is essential when two absolute differences of absorbance, differing by a very small figure, must be correlated with the analyte concentration [32]. The slope of the kinetic curve (ν) increases linearly with the concentration of OTA and is used as the analytical readout in all our experiments.

$$\nu = \frac{\nu_{max}[S]}{K_m + [S]} \overset{[S] \gg K_m}{\rightarrow} \nu = \frac{\nu_{max}[S]}{[S]} \Rightarrow \nu = \nu_{max} \quad (1)$$

For a sensor consisting of two hybridized oligonucleotides, the process of recognizing a small molecule is usually carried out after the hybridization process is complete [33]. In the case when the affinity between the small molecule and its aptamer is higher than that between the two oligonucleotides, the opening of the double-stranded oligonucleotides is possible. Compared with this protocol, in this new assay, the order of adding the reactants was different. We have observed that if we perform the hybridization between **N1** and **B2** before the addition of OTA, the low-molecular weight molecule could not be detected (Fig. 2). Therefore, we added OTA prior to the hybridization reaction. This behavior can be explained by the comparison of the different free Gibbs energies ($\Delta_r G_m^0$(298 K)) of the involved reactions. These values were calculated using the DINAMelt Web Server (freely available at http://mfold.rna.albany.edu/). It was found that with respect to a complete hybridization of **N1** with **B2,** the $\Delta_r G_m^0$(298 K) is -22.9 kcal mol^{-1}. Meanwhile, it is -12.5 kcal mol^{-1} for the hybridization between **B2** and 9 complementary bases corresponding to the DNAzyme part of **N1**, and -13.1 kcal mol^{-1} for the hybridization between **B2** and 9 complementary bases corresponding to the OTA aptamer part of **N1**. The dissociation constant of OTA and its aptamer is 200 nM and the $\Delta_r G_m^0$(298 K) of OTA and its aptamer is -9.2 kcal mol^{-1}. In conclusion, it is difficult for OTA to unlock the binding of **N1** and **B2**,

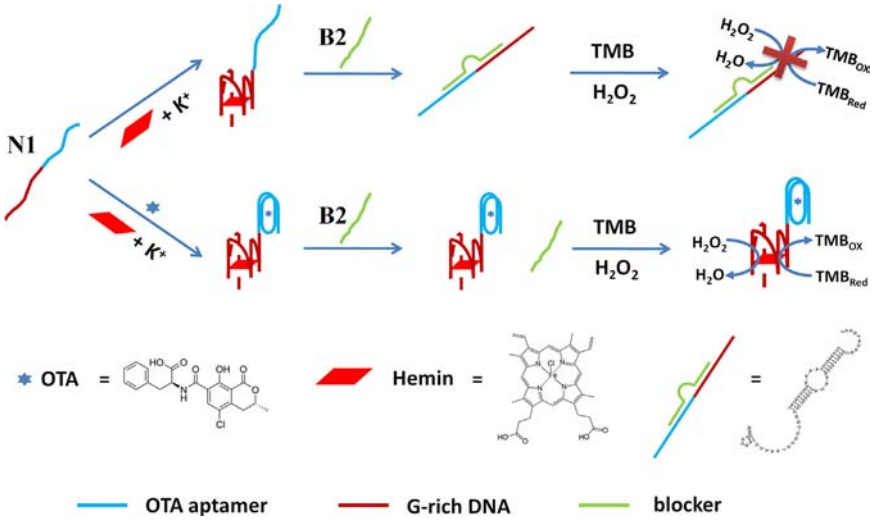

Fig. 1. Schematic illustrations of the principle of the bioassay.

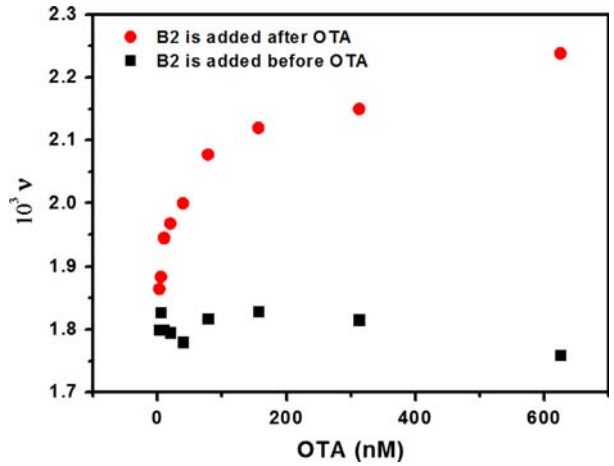

Fig. 2. Comparison of different protocols: ● **N1** reacts firstly with OTA and **B2** is added thereafter. ◆ Firstly **N1** and **B2** hybridize and OTA is added thereafter. Other experimental conditions: 50 nM of **N1**, 2.5 mM $MgCl_2$, 120 mM Na^+, 5 mM KCl, 100 nM hemin and 50 nM of **B2**. ν Is the slope of the kinetic curve.

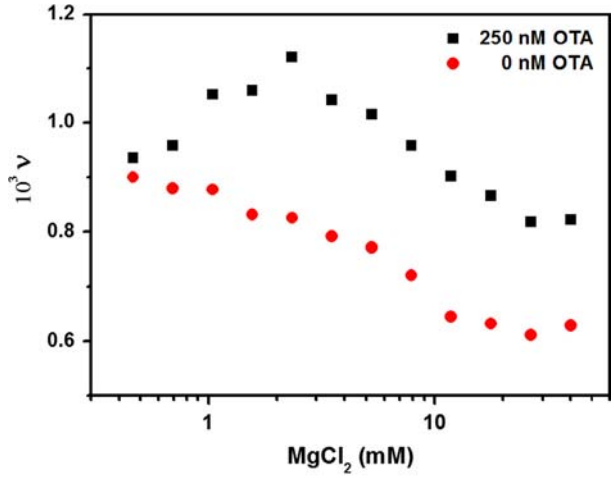

Fig. 3. Slope of the DNAzyme activity vs. concentrations of Mg^{2+} in the absence and presence of OTA. Experimental conditions: 5 mM K^+, 120 mM Na^+, 50 nM **N1**, 100 nM hemin, 50 nM **B2**.

regardless its concentration, due to the large energy of hybridization of **N1** and **B2**. This strategy could be used as a general method of detection for smaller affinity interactions between small molecules and aptamers. Due to the large energy of hybridization of N1 and B2, the reaction time between **B2** and **N1** is also important. With extended reaction time, the activity of DNAzyme decreased. This also proves that the binding of **N1** and **B2** is more stable than the **N1** binding with OTA and K^+. Therefore a reaction time of 10 min was chosen in this work.

3.2. Optimization of concentration of Mg^{2+}

As previously reported [17], the cofactor Ca^{2+} or Mg^{2+} is essential in the specific recognition of aptamer to OTA. We chose to work with Mg^{2+} and the optimum concentration was found by measuring the slope of DNAzyme in the presence and absence of 250 nM of OTA (Fig. 3). As shown in Fig. 3, in the absence of OTA, the enzyme activity decreased, with the increasing concentration of Mg^{2+}, due to the influence of the cation on the relative stability of the hybridization of **N1** and **B2**. The $\Delta_r G_m^0$ (298 K) of hybridization decreased as the ionic strength increased. In the presence of 250 nM OTA, the DNAzyme activity presented a maximum around 2.5 mM of Mg^{2+} proving that the presence of Mg^{2+} increased the affinity of OTA and its aptamer. Moreover, at low concentration of Mg^{2+}, the two curves overlap, which means that Mg^{2+} is required for OTA aptamer binding with OTA. So finally, the optimal concentration of Mg^{2+} in this assay was chosen as 2.5 mM.

3.3. Optimization of concentration of K^+

The stabilization of G-rich sequences of oligonucleotides into quadruplex structures by monovalent cations, especially K^+ was proved with thermodynamic and kinetic measurements [34]. Usually DNAzyme folds into G-quadruplex structure before binding to hemin [24]. Therefore, for the G-rich DNA sequence used in this experiment it can be expected that the enzymatic activity is K^+ dependent. The slope of the enzymatic activity increases with the concentration of K^+. In the presence of OTA, the enzymatic activity is higher, regardless of the concentration of K^+. Hence, as a compromise between sensitivity and ion strength, a concentration of 5 mM for K^+ was selected.

3.4. Colorimetric biosensing of OTA

To detect OTA using the self-assembly of DNAzyme–aptamer conjugates, the activity of the DNAzyme was measured via time-dependent absorbance changes in the presence of OTA in the concentration range of 0–220 nM, under the optimized experimental conditions.

As can be seen in Fig. 4, a linear correlation was obtained between the slope of the DNAzyme acitivty (ν) and the concentration of OTA from 4 nM to 30 nM, the regression equation being: $\nu = 0.00156 + 5.13 \times 10^{-6} C$ (C is the concentration of OTA (nM)) with the correlation coefficient of 0.982. A limit of detection (LOD) of 4 nM (3σ) was obtained. This LOD is comparable to that of the analytical method based on HPLC-FD [4] and satisfies requirements for quantitative measurement of OTA in wine and grape-based beverages. The specificity of the assay was checked by replacing OTA with OTB and warfarin. As can be seen in Fig. 4, no significant change in the DNAzyme activity is recorded in a wide range of concentration of OTB and warfarin, proving that the assay is specific for OTA detection.

3.5. Principle of new method for extraction and purification of OTA from red wine using double LLE

Extraction of OTA from wine using a suitable solvent is an important step to achieve highly-performing analytical measurements. Toluene and chloroform have been used during the process of extraction and clean-up for their good extraction ability [35,36]. The pK_a values of OTA are in the ranges of 4.2–4.4 and 7.0–7.3, for the carboxyl group of the phenylalanine moiety and the phenolic hydroxyl group of the isocoumarin part, respectively. The pH of red wine is 3–4, and at this pH, OTA dissolves in polar organic solvents. If we add toluene or chloroform to wine samples, OTA is easily extracted to the organic phase. Then, if we mix these organic phases with Tris–HCl buffer (pH = 8.4), OTA dissolves in Tris–HCl buffer, because at this pH OTA dissolves in alkaline aqueous solution. However, the sample extracted with toluene or chloroform and Tris–HCl buffer does not fit for the colorimetric bioassay based on OTA aptamer and DNAzyme, because wine is an aqueous matrix with very complex composition and some substances which have similar solubility as OTA are extracted as well, and these substances may influence the catalytic activity of DNAzyme. In addition, as shown in Fig. 5, the spectra with chloroform treatment is different from that with toluene (line A and B respectively), which means that these two organic solvents can clean different impurities of wine. Therefore, we combine the two methods together. From curve C in Fig. 5, we can see that the

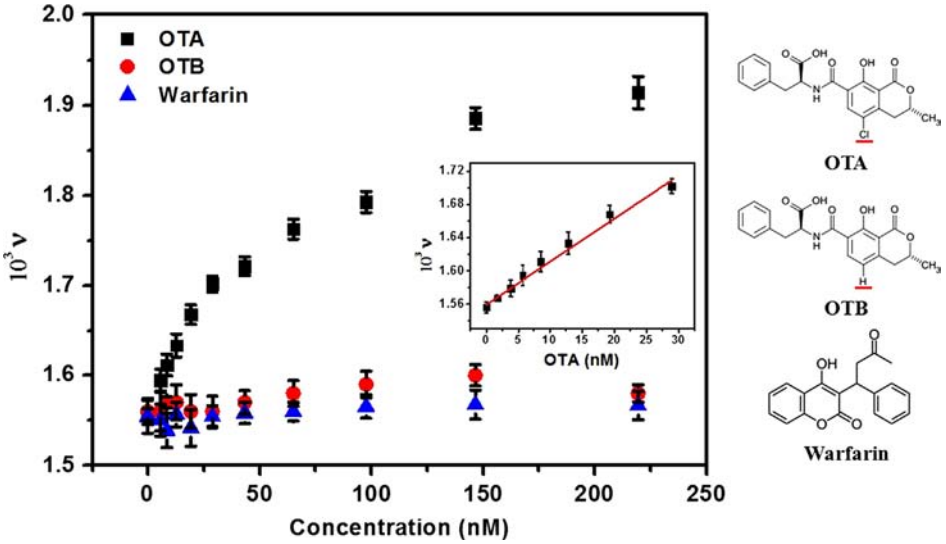

Fig. 4. Calibration curve for OTA derived from the enzyme activity (slope) of the aptamer–DNAzyme structure. Experimental conditions: 2.5 mM Mg^{2+}, 120 mM Na^+, 5 mM K^+, 50 nM **N1**, 100 nM hemin, 50 nM **B2**. Error bars are standard deviations of the mean with $n=3$.

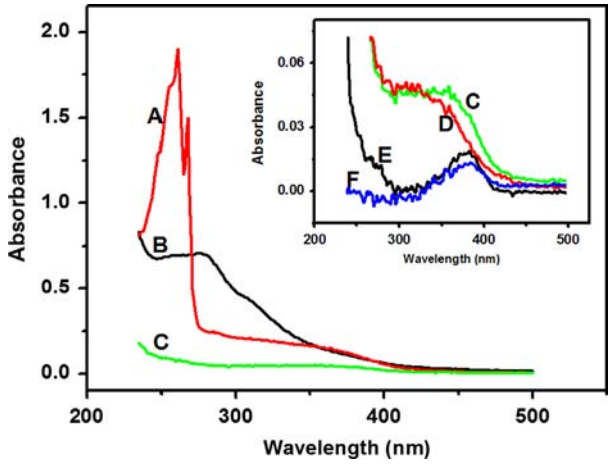

Fig. 5. UV–vis absorption spectrum. A–C are spectrums of wine sample containing 1250 nM OTA after treatment with chloroform, Toluene and double LLE, respectively. D is wine sample without OTA after treated with double LLE. E is 1250 nM OTA in Tris–HCl buffer (pH=8.4). F is difference between C and D.

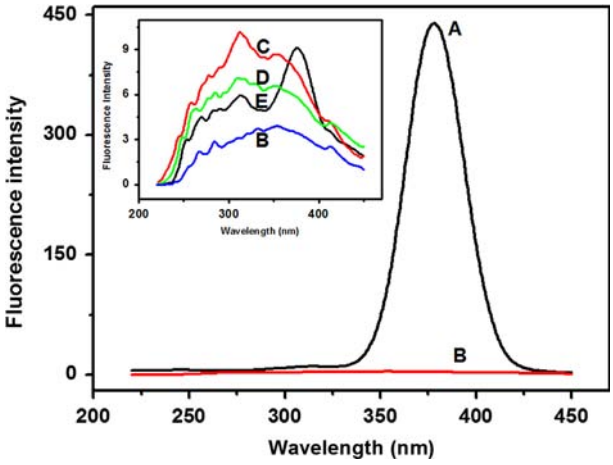

Fig. 6. Synchronous fluorescence spectroscopy: (A) wine sample containing 1250 nM OTA after treatment with double LLE, (B) Tris–HCl buffer (pH=8.4), (C) wine sample after treated with double LLE, (D) wine sample after treated twice with double LLE, and (E) wine sample containing 1250 nM OTA after treated twice with double LLE. ($\Delta\lambda=130$ nm).

UV–vis absorption spectrum for wine sample after treatment with double LLE is obviously lower than the sample just treated with chloroform or toluene. An enlargement of this spectrum is illustrated in the inset of Fig. 5. The spectrum obtained as a difference (line F) of the two UV–vis absorption spectra (line C and D) is similar to the spectrum of 1250 nM OTA in Tris–HCl buffer (line E). This means that the double LLE is able to clean-up the wine samples. Here, we borrow the concept of the distribution coefficient to calculate how many OTA remains in the original wine sample after treatment with double LLE. For this, synchronous fluorescence spectroscopy was used as shown in Fig. 6. Here we have defined distribution coefficient as the ratio of OTA that was extracted in the first time to that in the second time; this values were corrected for the background signal of the wine sample without OTA. The equation was as follows:

$$\text{Distribution coefficient} = \frac{\text{A}-\text{C}}{\text{E}-\text{D}} = \frac{439.87-7.33}{9.04-5.75} = 131.48$$

The distribution coefficient is 131.48, this means less than 1% of OTA remained in original wine sample.

3.6. Analysis of OTA in wine samples

OTA was extracted from wine using the extraction protocol described in experimental Section 2.4. Then we used the colorimetric biosensing by the self-assembly of DNAzyme–aptamer conjugates to detect the concentration of OTA. A calibration curve with OTA extracted from wine samples was obtained: $\nu=0.00196+9.25\times10^{-5}\log C$ (C is the concentration of OTA (nM), the linear range is 1.2–40 nM), $R^2=0.997$. A limit of detection of 4 nM (3σ) was achieved (Fig. 7). However, the specificity of our double LLE method is presumably lower than that of an affinity column because this method is based on the physical property of OTA. And there are inevitably low concentrations of some impurities in the final solution even after the double LLE. This is verified in the UV–vis absorption spectrum. Nevertheless the OTA aptamer has very good selectivity and affinity toward OTA and these impurities will not influence the binding aptamer with OTA, but they may probably influence the activity of DNAzyme. All these could explain the logarithmic but not linear variation of the

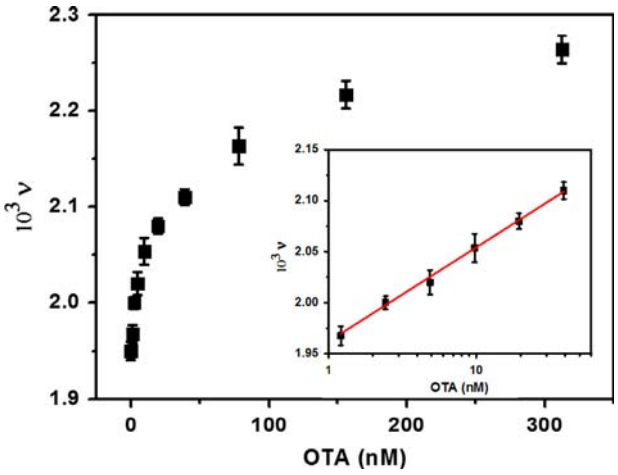

Fig. 7. Calibration curve for OTA in wine derived from the DNAzyme activity (slope) of the aptamer–DNAzyme structure. Other experimental conditions: 50 nM of **N1**, 2.5 mM $MgCl_2$, 120 mM Na^+,5 mM KCl, 100 nM hemin and 50 nM of **B2**.

Table 1
The determination of OTA by colorimetric bioassay in spiked wine.

OTA added, nM	OTA found, nM ($n=3$)	Recovery (%)
40.0	41.9 ± 1.2	104.8
19.5	19.8 ± 1.2	101.5
9.8	10.3 ± 1.4	105.4

DNAzyme activity (slope of kinetic measurements) with the concentration of extracted OTA. Despite this, a good assay recovery for spiked real wine samples was obtained as shown in Table 1.

3.7. Performance comparisons with other sensors reported in the literature for OTA detection

The performance of the developed colorimetric bioassay for the detection of OTA was compared with the other reported sensors for OTA detection. Immunosensor-based strategy for OTA has been developed by Prieto-Simon, et al. [9]. They compared two indirect competitive enzyme-linked immunosorbent assay (ELISA) strategies and electrochemical immunosensors based on different enzyme-labelled secondary antibodies. The value of IC_{50} was 41.3 and 13.9 ng/mL (equivalent to 103.25 and 34.75 nM) for HRP- and ALP-labelled ELISA respectively. The LOD was 0.7 and 0.3 ng/mL (equivalent to 1.75 and 0.75 nM) for HRP- and ALP-labelled immunosensors respectively. Electrochemical aptamer-based biosensors for OTA have been described by Castillo et al. [19], the LOD value of the assay was 1 nM and the effective sensing range was 1–30 nM. Also, a fluorescence aptasensor based on a target-induced structure-switching signaling aptamer was developed. This aptasensor showed a wide linear range from 2.5 nM to 250 nM of OTA with a detection limit of 2 nM [21]. The performances of our developed bioassay are comparable with these immunosensors and aptasensors reported for OTA detection. Moreover, this bioassay is a label-free colorimetric biosensing assay where no label was conjugated with the oligonucleotide. Another advantage of the proposed assay is given by the new extraction method of OTA from wine samples. The new proposed purification method is based only on the physical and chemical properties of OTA. It does not require any affinity column (with antibody or aptamer) which reduces the cost substantially. Compared to the impedimetric detection, the optical detection requires less expensive devices. Overall, the proposed assay offers a reduced labor and less time-consuming protocol.

4. Conclusions

We have developed a new colorimetric bioassay for the detection of OTA on the basis of self-assembly of DNAzyme–aptamer conjugates. The structure of the new DNA includes the OTA-specific aptamer and a G-rich sequence of nucleotides mimicking peroxidase activity. The binding of OTA to aptamer results in a decrease of the hybridization efficiency, leading to an increase of the DNAzyme activity. It is shown that the activity of DNAzyme is influenced also by the concentration of several species like Mg^{2+} and K^+. At the same time Mg^{2+} is essential for the recognition of OTA by the aptamer. Consequently, the optimal concentration of Mg^{2+} was selected in order to obtain a high sensitivity. The activity of DNAzyme was correlated well with OTA concentration in a linear range up to 30 nM. The limit of detection (4 nM) meets the requirement of OTA screening in wine samples. The significance of this paper is that we not only present a new bioassay concept, but also provide a complete set of implementation approaches. A facile and reliable double LLE method was developed to extract OTA from practical wine samples. The present method exhibited impressive efficiency and high-throughput (less than 1 h for 96 samples). It may eventually become a simpler, faster and more economical complement to the current standard HPLC-FD method coupled with immunoaffinity column clean-up. The practicability makes the assay to hold great potential for the preparation of test kits.

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