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An indirect competitive assay-based aptasensor for detection of oxytetracycline in milk

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ABSTRACT

Oxytetracycline (OTC) is a common antibacterial agent used for the control of animal diseases. OTC abuse can seriously affect human health; therefore, we developed a biosensor using single-stranded DNA (ssDNA) aptamers for the detection of OTC. The binding probe aptamers for OTC were selected by a Systematic Evolution of Ligands by the exponential enrichment (SELEX) process and identified by the enzyme-linked aptamer assay (ELAA). Among the selected 5 aptamers, aptamer OTC3 showed the strongest affinity (K_d=4.7 nM) and highest specificity for OTC compared to structurally similar antibiotics, tetracycline and chlortetracycline. OTC was detected using indirect competitive ELAA. The limit of detection and quantitation with aptamer OTC3 were 12.3 and 49.8 μg/L, respectively, in milk and showed recovery rates of more than 90% in OTC-spiked milk. This biosensor method with high sensitivity and specificity based on indirect competitive ELAA can be applied to OTC detection in food products on-site because of the simplicity of detection.

1. Introduction

Oxytetracycline (OTC) is a member of the broad-spectrum tetracycline (TC) group of antibiotics. TCs are widely used to prevent bacterial infections in livestock and increase their growth rate. Abuse of TCs in farm animals can cause accumulation of antibiotics in food products, including meat, milk and chicken eggs (Cinquina et al., 2003; De Wasch et al., 1998; Furusawa, 1999). Ultimately, this accumulation is likely to have serious implications for human health. Antibiotic residues in milk can trigger the emergence of antibiotic-resistant bacteria (Kitazono et al., 2012) therefore, several countries have set maximum residue limits (MRLs) for many food products (Casella and Picerno, 2009), and extensive efforts have been made to develop a sensing system for the enhanced detection of antibiotics in contaminated food products (Mehta et al., 2011; Niazi et al., 2008a; Song et al., 2012).

Traditionally, chromatography methods, including high-performance liquid chromatography (HPLC) and liquid chromatography-tandem mass spectrometry (LC–ESI-MS/MS), have been used for the detection of TCs in food products (Lv et al., 2012; Boscher et al., 2010). Although these methods provide simultaneous and accurate detection of TCs, they demand expensive equipment, tedious sample extraction procedures, and expert technical skill. Moreover, immunochemical methods using antibody have been used owing to simplicity and cost-effectiveness and detection with high sensitivity and specificity (Pastor-Navarro et al., 2007), but the use of an antibody as a probe for detection has drawbacks in terms of stability and production.

Aptamers are short single-stranded oligonucleotides with a three-dimensional structure that show high affinity binding and high-specificity target recognition (Jenison et al., 1994; Shangguan et al., 2007). Aptamers have a number of advantages over antibodies because they are small and can be raised against any type of target, including toxic compounds or poor immunogenic targets (Bruno et al., 2009), in a reproducibly synthesized and stabilized manner (Ahmad et al., 2011; Juskowiak, 2011). In addition, a variety of derivatives such as labeled molecules can be conveniently attached at the 3′ or 5′ end of an aptamer without affecting the target-binding site (Huang et al., 2010; Pultar et al., 2009). In recent years, many biosensors using ssDNA aptamers selected by the Systematic Evolution of Ligands using the EXponential enrichment (SELEX) process have been reported for various small molecular targets (Ding et al., 2009; Reinemann et al., 2009; White et al. 2001). Aptamers for several antibiotics belonging to the TC class have been used for the development of biosensors for the detection of TCs in many food products from animals. Electrochemical aptasensor showed a lower limit of detection (LOD, 1 ng/mL) and short detection time (Zhang et al., 2010). However, this sensing system required a special instrument for signal detection, and the immobilization procedure of the aptamers on an electrode is time

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consuming. Colorimetry has been proposed as a simple technique for the detection of signaling with the naked eye on-site. Despite the convenience, specificity, and sensitivity, detection of OTC in real food samples has not yet been applied. In addition, the observed LOD (5 μM) by naked eye was not significantly higher than the LOD (25 nM) by UV/vis spectrophotometer analysis for OTC detection (Kim et al., 2010; Kim et al., 2011).

In this study, we performed an indirect competitive enzyme-linked aptamer assay using a biotin-labeled aptamer for the detection of OTC in milk for the first time. This proposed method offers good sensitivity (LOD=27 nM in milk) similar to colorimetric aptasensor with high specificity and stability, and does not involve complicated sample extraction steps.

2. Materials and methods

2.1. In vitro selection of aptamers for OTC

A random ssDNA library containing 40 random nucleotides, flanked by invariant primer annealing sites (5′-CGTACG-GAATTCGCTAGC-N40-GGATCCGAGCTCCACGTG-3′), was used for the initial pool. Three different primers were used for PCR amplification: aptF, 5′-CGTACGGAATTCGCTAGC-3′; aptR, 5′-CACG-TGGAGCTCGGATCC-3′; and aptRBioT (equivalent to aptR with 5′ Biotin). Prior to use, 10 μM of the random DNA library pool was dissolved in 100 μL of binding buffer (20 mM Tris–HCl, 50 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, pH 7.2) and denatured at 95 °C for 10 min, quickly cooled at 4 °C for 5 min, and then placed at RT for 5 min.

Selection of aptamers for detecting OTC was performed using a SELEX method. First, the OTC–BSA conjugate (OTC–BSA conjugate for aptamer selection was purchased from Glory Science Co., TX, USA) or BSA was adsorbed onto the wells of microtiter plates. The coating step was performed in 50 mM carbonate buffer, pH 9.6. The plates were coated with 100 μL/well of 5 μg/mL of OTC–BSA conjugate or BSA solution and incubated overnight at 4 °C. The incubated plates were covered with 200 μL/well of 1% (w/v) BSA solution prepared in the binding buffer for 60 min at 37 °C, and unbound BSA was removed. The denatured ssDNA library pool was then added to the BSA-coated wells to eliminate the candidates capable of nonspecific binding of BSA. The unbound ssDNAs were collected and added to the OTC–BSA coated wells for 60 min at 37 °C. After incubation, the unbound and weakly bound ssDNAs were removed and washed 3 times with PBS containing 0.01% Tween-20 (PBST). The bound ssDNAs to OTC were incubated in 100 uL of 20 mM NaOH at RT for 10 min with mild shaking, and the ssDNAs were eluted. The process was repeated 2 times to elute the bound ssDNAs. The eluted oligonucleotides were precipitated by ethanol precipitation and the ssDNAs were dissolved in a final volume of 10 μL of EB buffer (10 mM Tris–HCl, pH 8.5). To monitor the progression of SELEX, the amount of eluted ssDNAs from OTC–BSA-coated wells was measured by Nanodrop device. The eluted ssDNA fraction was amplified by PCR (1 cycle at 95 °C for 5 min; 35 cycles at 95 °C for 30 s, 48 °C for 30 s, and 72 °C for 30 s; followed by 1 cycle at 72 °C for 5 min), using a biotinylated reverse primer (aptRBioT) that allowed the subsequent purification of the aptamer strand by alkali denaturation on Dynabeads streptavidin M-280 (Wochner et al., 2007). The amount of separated ssDNAs was measured using a Nanodrop device, and the separated ssDNAs were used for the next rounds for further enrichment of aptamers. The selection procedure was repeated until the twelfth round. After the last round, the eluted ssDNAs were amplified with unmodified primers aptF and aptR, and cloned into the TOPO PCRII plasmid vector (Invitrogen). Aptamer inserts of 12 candidates were then sequenced.

2.2. Binding assays of aptamers for OTC

Sequenced OTC-specific aptamers were synthesized as biotinylated aptamers and tested for their individual characteristics for binding to OTC. These aptamers were also verified for their specific binding affinity using OTC–BSA-conjugate or BSA-coated plates. The random oligonucleotide (Oligo) library was used as the negative control. First, biotinylated aptamers were heated at 95 °C for 10 min, cooled at 4 °C for 5 min, and placed at RT for 10 min. Each aptamer (5 nM) was then incubated for 60 min at 37 °C in BSA-coated or OTC–BSA coated wells of microtiter plates blocked by 1% (w/v) OVA in the binding buffer for 60 min at 37 °C. After the binding reaction, a streptavidin–HRP (0.5 ng/mL) solution was added and allowed to react (30 min, 37 °C). In the final step, 100 μL of TMB solution was added to measure the absorbance after 30 min at 450 nm (after quenching with 100 μL/well of 1 N H_2SO_4). Each step was performed with constant shaking and protection from light. The wells were thoroughly rinsed 3 times with PBST between each step. The assays were performed in triplicate.

The K_d of the aptamers OTC3, OTC6, OTC9 and OTC12 were determined. Binding reactions were conducted as described above, but with increasing amounts of biotinylated aptamers (1–100 nM). The amount of OTC-bound aptamers was determined by absorbance measurement at 450 nm and converted into the corresponding percentage of binding. The value was determined as follows: $(A/A_{100})\% = (A/A_{100}) \times 100$, where A is the absorbance value of the amount of aptamer with a concentration ranging from 1 to 100 nM and A_{100} is the absorbance value of 20 nM of aptamer OTC3. K_d was calculated by nonlinear regression analysis.

2.3. Indirect competitive enzyme-linked aptamer assay (ic-ELAA)

The indirect competitive OTC binding assay was performed as previously described. Competition was allowed to proceed by adding nonlabeled OTC and biotinylated aptamers. Solutions were prepared in the binding buffer, and the competition time was 60 min at 37 °C. After the competitive reaction, a streptavidin–HRP solution was added and allowed to react. Finally, 100 μL of TMB solution was added, and the absorbance was measured at 450 nm. The assays were performed 5 times. The absorbance values were converted into their corresponding test inhibition values (A/A_0) as follows: $(A/A_0)\% = (A/A_0) \times 100$, where A is the absorbance value for the competitive assay and A_0 is the absorbance value for the noncompetitive assay.

2.4. Specificity of the OTC aptamer for TCs

Analysis of the specificity of OTC aptamers for TCs was performed in the same way as the ic-ELAA method. TC and chlortetracycline (CTC), which are structurally similar to OTC, were used as cross-reactants, and the presence of cross-reactivity was calculated by determining the inhibition values of the competitive assay.

2.5. Pretreatment for the analysis of milk samples

Milk samples were purchased from local supermarkets and stored at 4 °C before use. Milk contains protein, fat, carbohydrate and cations such as Ca^{2+} and Mg^{2+}, which can form chelation complexes with OTC. To remove these components, milk samples (5 mL) were mixed with 5 mL of McIlvaine buffer containing 20 mM EDTA (pH 5.0) and 0.5% (v/v) trifluoroacetic acid to denature milk proteins. The milk samples were then defatted and deproteinized by centrifugation at 4 °C for 20 min at 8000 rpm. Then, 1 M NaOH was added dropwise to the supernatant to adjust the pH to 7.0. The supernatant fluid was filtered

through a 0.45-μm membrane filter (Corning, Germany) and evaporated to dryness by using a gentle nitrogen blow-down at 40 °C. After this extraction, the precipitate was resuspended with the binding buffer, and OTC was added to the treated milk samples. The detection of OTC in milk samples was validated by ic-ELAA with the aptamer OTC3, as described above. The HPLC-UV system for OTC analysis was also used as previously described (Casella and Picerno, 2009).

3. Results and discussion

3.1. Selection of specific aptamers for OTC

ssDNA aptamers against OTC was selected by the SELEX procedure using OTC–BSA conjugate (Fig. S1). The progress of the selection process was confirmed by the recovery rate of ssDNAs from OTC–BSA-coated wells. As shown in Fig. S2, the recovery rate of ssDNAs bound to OTC increased in small quantities until the fourth round (1.5%); however, the rate increased gradually by the fifth round (4.6%) and then increased rapidly by the ninth round (27%). This result indicates that the nonspecific or BSA-bound ssDNAs were discarded during the progression of SELEX. After the ninth round, no increase was observed. This fact indicates the saturation of the binding affinity sites for OTC. Because BSA is larger than OTC, our SELEX using an OTC–BSA conjugate may be problematic because of the possibility of covering up of the target molecule or the binding epitope for ssDNA. Nevertheless, this screening method can select aptamers with a high binding affinity for OTC.

Based on sequence analysis, 5 aptamers from the twelfth round of selection that had been cloned were identified and then screened for OTC-binding affinity. The 5 selected aptamers were designated OTC3, OTC6, OTC9, OTC12, and OTC16. Among these candidates, 4 aptamers were found to show affinity on OTC–BSA-coated plates with a distinct specificity for OTC compared with their affinity on BSA-coated plates (Fig. 1). Specifically, the OTC3 and OTC9 aptamers showed higher binding capacity for OTC than the other aptamers did.

Analysis of the variable 40-nucleotide (N_{40}) region of the selected aptamers for OTC by ClustalW showed highly conserved consensus sequence motifs of AGGGA or AGGA and several sequence motifs (Table 1). These consensus sequence motifs are palindromic sequences, and the palindromic sequence motifs of these aptamers have been reported to be crucial for binding to TCs (Niazi et al., 2008a). In particular, the CGCTGGTG sequence motif of aptamer OTC3 has been reported to be a significant motif for OTC binding (Niazi et al., 2008b), and the OTC3 aptamer showed the highest binding for OTC compared with the other aptamers (Fig. 1).

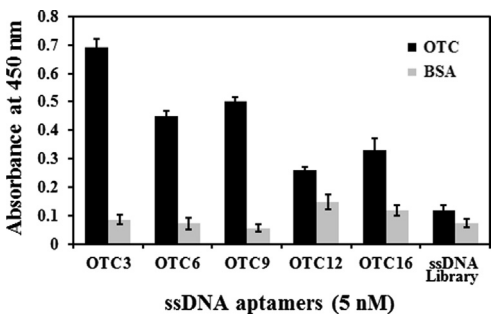

Fig. 1. Binding affinities of selected aptamers for oxytetracycline (OTC) on OTC–BSA-conjugate or BSA-coated plates. Binding assays with the selected aptamers, OTC3, OTC6, OTC9, OTC12, and OTC16, were performed using the ELAA method. The ssDNA library was used as the negative control. Error bars represent standard deviation of the mean, with determination of 3 ($n=3$).

Table 1
Variable N_{40} sequences of the 5 aptamers selected for OTC. The conserved sequences of each aptamer are indicated in bold type and binding sequence motifs of TCs are underlined.

Aptamers	Sequences 5′→3′
OTC3	CGACGCACAGTCGCTGGTGCGTACCTGGTTGCCGTTGTGT
OTC6	GGCGA**AGGA**GTCATGTAGGTGTGGTCGAGACCGCTGTGCT
OTC9	GGCGCCGGCATGGTGTGGACTCCAGGCGGT**AGGGA**TGTCGT
OTC12	AC**AGGGA**GCGCAAGAATACGACCCATATCTCCCCCGTGCT
OTC16	GAA**AGGGA**CGTTCCAAGTTCGTATAAGCAGTCCTGTGCGT

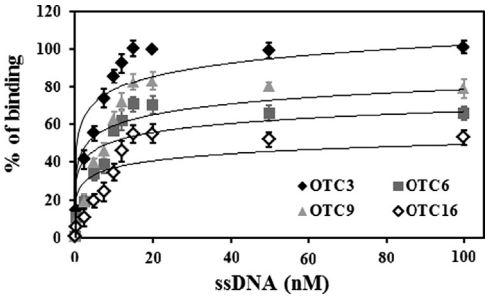

Fig. 2. Binding affinities of the aptamers OTC3, OTC6, OTC9, and OTC16. The saturation curves were obtained by the binding percentage of aptamers for OTC as described in materials and methods. The dissociation constants (K_d) of aptamers were calculated by nonlinear regression analysis. Error bars were standard deviation of the mean, with determination of 3 ($n=3$).

In addition, the secondary structures of OTC aptamers were characterized using the m-fold program. Their secondary structures showed stem and loop structures that may be binding sites of the aptamers. As shown in Fig. S3, The highly conserved sequence motifs (AGGGA and AGGA) and the other sequence motifs were located mainly in the stem and loop region of each aptamer. The aptamers OTC3, OTC6, OTC9, and OTC16 have a very similar structure. However, OTC12 has a substantially different structure, and showed the weakest binding to OTC of all the selected aptamers, with a higher affinity for BSA (Fig. 1 and S3). The specific location of this conserved sequence motif in the secondary structure of aptamers for OTC had an important effect on the binding capacity and specificity for the target molecules, for example, OTC.

3.2. Binding characteristics of the selected aptamers for OTC

The ELAA method was used to compare the binding affinity of the selected aptamers for OTC. To determine the optimal working condition of OTC and aptamers, immobilized OTC–BSA used at different concentrations (1, 2, 5, and 10 μg/mL) was exposed to various concentrations of biotinylated aptamers. Saturated coating of wells was achieved using 5 μg/mL of the OTC–BSA conjugate. The concentration of streptavidin–HRP was found to be 0.5 μg/mL. Binding reactions were conducted using a fixed quantity of OTC–BSA conjugate against varying concentrations of the selected aptamers (1–100 nM). The K_d values of 4 aptamers, that is, OTC3, OTC6, OTC9, and OTC16, were determined by nonlinear regression analysis of the binding data to determine binding affinity (Fig. 2). The K_d values of the 4 aptamers were in the nanomolar range. The lowest K_d value (4.7 nM) was obtained with the aptamer OTC3. The other 3 aptamers, that is, OTC6, OTC9, and OTC16, showed higher K_d values, that is, 9.5, 8.0, and 14.0 nM, respectively, than the aptamer OTC3. The differences in the binding capacity between these aptamers could be related to the effect of ionic

interactions along with their differing sequences and three-dimensional structure (Girardot et al., 2010).

3.3. Detection of OTC by indirect competitive ELAA

The indirect competitive assay depends on both the immobilized OTC–BSA concentrations and the competitor concentrations of aptamers. In the indirect competitive assay, the coating concentration of the OTC–BSA conjugate on well plates with the same concentration (5 μg/mL) as that used in the binding assay involving aptamers for OTC and 3 concentrations of the aptamer OTC3 (2, 5, and 10 nM) were used to investigate the sensitivity for OTC. The concentration of free OTC resulting in 50% inhibition (IC_{50}) of binding to the immobilized OTC was determined for 5 and 10 nM of aptamers, which was approximately 4.5 and 13.5 fold higher, respectively than the IC_{50} value obtained for 2 nM (Fig. 3A). The 2 nM concentration of the aptamer OTC3 was found to provide optimum sensitivity and sufficient absorbance values for an easily detectable signal. The results demonstrated that sensitivity increased when lower concentrations of aptamers were used. Similar results have also been observed in a competitive assay involving an aptamer for ochratoxin (Barthelmebs et al., 2011). Fig. 3B showed the dose–response curve constructed under optimized conditions by using 2 nM of aptamer OTC3 and 5 μg/mL of OTC–BSA in each well/plate. The calibration curves of these aptamers were calculated as follows: (1) binding (%) of OTC3 in buffer $= -22.25 \times \ln[\text{OTC}] - 12.71$, ($R^2 = 0.99$); (2) binding (%) of OTC3 in milk $= -21.73 \times \ln[\text{OTC}] - 7.96$, ($R^2 = 0.99$). The limit of detection (LOD) and limit of quantitation (LOQ) of aptamer OTC3 in buffer at 10.1 and 33.4 μg/L, respectively, showed the best sensitivity for OTC. In addition, the LOD and LOQ of OTC3 in milk were 12.3 and 49.8 μg/L, respectively. These results indicate that the ic-ELAA method using aptamer OTC3 yields similar LOD values in buffer and milk without the matrix effect. The LOD and LOQ are defined as the concentrations corresponding to 3 standard deviations or 10 standard deviations below the mean from the blank, respectively.

Recently, a competitive ELAA using 2 different aptamers was developed to detect TC in milk (Jeong and Rhee Paeng, 2012). The developed method showed a good LOD (DNA aptamer was 42.3 and RNA aptamer was 15.6 ug/L), but low specificity for TC detection in milk. Although the target for detection differs, our proposed method indicates excellent sensitivity and specificity compared to other methods.

3.4. Specificity of the aptamers for OTC

In the aptamer assay, the ability of an aptamer to selectively bind to the target molecule is described as specificity (Low et al., 2008). The specificity of the aptamer with high binding for OTC was evaluated by cross-reactivity. Cross-reactivity was determined using the ic-ELAA with the structurally similar compounds TC and CTC. Their cross-reactivity rates were calculated using a previously described method (Jeon and Rhee Paeng, 2008). The 50% $B/B0$ value and cross-reactivity for each compound are shown in Fig. 4 and Table S1. These 2 reactants (TC and CTC) showed different cross-reactivities of 12.7% and 7.9%, respectively. The aptamer OTC3 showed low cross-reactivity for TC and CTC. These results suggest that the OTC-specific motif, CGYTGGTG of aptamer OTC3, may be crucial for binding at carbon atoms 5 and 6 (–OH and –OH positions) on aromatic rings, which are distinct between OTC structure and the common TC backbone (Niazi et al., 2008a).

3.5. Detection of OTC in milk by using indirect competitive ELAA and HPLC

The effectiveness of the ic-ELAA method for detecting OTC was validated using a sample that was treated with an OTC. The recovery study of the sample was performed using 2 different methods, that is, ic-ELAA and HPLC-UV. The samples were prepared by extraction involving OTC spiked in milk at 0.5,1, 2, and 4 times the MRL. The recovery rate of OTC in extracted milk samples was analyzed simultaneously by the ic-ELAA and HPLC-UV methods, and concentrations of OTC obtained by the two methods were compared. Average recovery rate of OTC spiked milk when detected by ic-ELAA and HPLC-UV was 93.6% and 94.8%, respectively. Both methods showed a recovery rate of $\geq 90\%$ for OTC in milk, and the relative standard deviation (RSD) was less than 5% (Table 2). The recovery study was performed in triplicate. These results validate the use of ic-ELAA and HPLC-UV as satisfactory screening methods. This biosensor can effectively detect OTC in samples under practical circumstances with sufficient sensitivity.

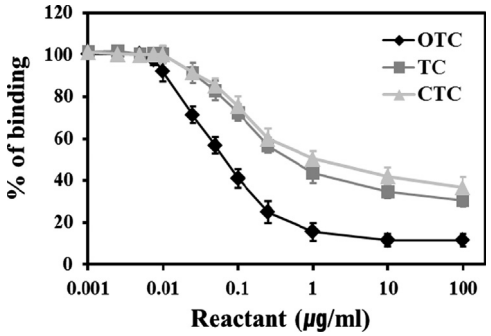

Fig. 4. Cross-reactivity of aptamer OTC3 (2 nM) with OTC, TC, and CTC. Error bars were standard deviations of the mean, with determination of 5 ($n=5$).

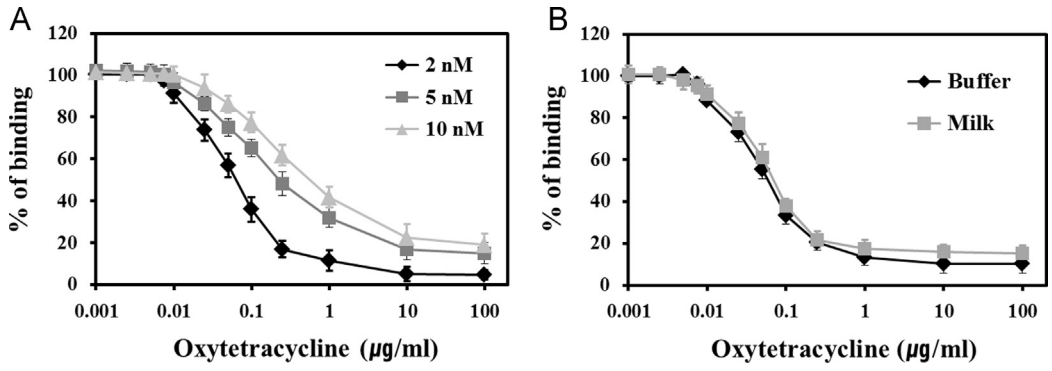

Fig. 3. Indirect competitive ELAA. (A) Calibration curves for OTC by indirect competitive ELAA, using various concentrations of the aptamer OTC3. (B) Competition curves obtained using 2 nM of the aptamer OTC3 in buffer solution and milk. Error bars were standard deviations of the mean, with determination of 5 ($n=5$).

Table 2

Methods	Spiked concentration (μg/L)	Measured concentration (μg/L, mean ± SD)	Recovery (%, mean)	RSD (%)
ic-ELAA	50	43.7 ± 1.5	87.4	3.6
	100	101.3 ± 2.1	101.3	2.1
	200	186.5 ± 4.9	93.2	2.6
	400	364.1 ± 8.8	91.0	2.4
HPLC-UV	50	47.4 ± 1.1	94.7	2.3
	100	93.4 ± 1.2	93.1	1.3
	200	190.9 ± 4.7	94.8	2.5
	400	377.6 ± 7.2	94.4	1.9

※ SD; Standard deviation ($n=3$).
RSD; Relative standard deviation.

4. Conclusions

To detect OTC in milk, we developed the indirect competitive assay-based aptasensor. The OTC3 aptamer, selected by the SELEX procedure using OTC–BSA conjugate was used as an aptasensor probe. The OTC3 had the best affinity ($K_d=4.7$ nM) among the selected aptamers, with high specificity for OTC. In this study, the proposed indirect competitive ELAA method showed a low LOD of 12.3 μg/L that could be compensated for a required MRL (100 ug/L in Codex) in milk, and a high recovery rate of more than 90% in OTC spiked milk. With high sensitivity and specificity, this selected aptamer along with the established method will provide a simple screening tool for on-site monitoring of OTC levels in food products, including milk.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.08.003.

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