Aptamer assisted ultrafiltration cleanup with high performance liquid chromatography-Fluorescence Detector for the determination of OTA in green coffee.

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Abstract:

Ochratoxin A (OTA) has a chronic toxic effect and proved to be mutagenic, nephrotoxic, teratogenic and carcinogenic. Several methods have been reported for the determination of OTA in foods. However, most of these methods could not be applied to a complex food as green coffee because the interfering native fluorescent products made the quantification very difficult. In this work, we mixed two separations based techniques to identify and quantify OTA in green coffee. Aptamer assisted ultrafiltration as separation technique was applied to separate the free OTA, the quantification of OTA was established by a high-performance liquid chromatography (HPLC-FD) with a limit of detection (LOD) of 0.05 ng/mL for OTA. Artificially contaminated green coffee displayed a good range of OTA recoveries up to 97.7%. This method can be applied to the quantitative determination of OTA in green coffee.

Keywords: Ochratoxin A, Aptamer, ultrafiltration, Green coffee, HPLC-FLD.

Abstract

One of the most common mycotoxin contaminating feed and foodstuffs is Ochratoxin A (OTA). OTA has a chronic toxic effect and proved to be mutagenic, nephrotoxic, teratogenic, immunosuppressive, and carcinogenic. Aptamer with their specific affinity for OTA was used in this paper to create an analytical technique. Several methods have been reported for the determination of OTA in foods. However, most of these methods could not be applied to a complex food as green coffee because the interfering native fluorescent products made the quantification very difficult. In this work, we mixed two separations based techniques to identify and quantify OTA in green coffee. Aptamer assisted ultrafiltration as separation technique based on the size of molecules was applied to separate the free OTA, the quantification of OTA was established by a high-performance liquid chromatography (HPLC-FD) with a limit of detection (LOD) of 0.05 ng/mL for OTA. Artificially contaminated green coffee displayed a good range of OTA recoveries up to 97.7%. This method can be applied to the quantitative determination of OTA in green coffee at levels below the maximum levels proposed by the European Commission for green coffee. It also confirm that aptamers can be used as biorecognition element in diagnostic assays with commercial application for mycotoxin analysis.

Keywords: Ochratoxin A, Aptamer, ultrafiltration, Green coffee, HPLC-FLD.

1. Introduction

Governments and international instances are trying to improve the food security system to prevent, reduce or avoid the increase of food borne diseases. This food risk is one of the major concerns for the humanity. The contamination by mycotoxins is a threat to the health and life of humans and animals. Among mycotoxins, ochratoxin A (OTA) (Figure 1) represents one of the most widespread and hazardous substances (Giovannoli, Passini, Di Nardo, Anfossi, & Baggiani, 2014; Shephard, 2008). It is a secondary metabolite produced by Aspergillus (Van der Merwe, Steyn, Fourie, Scott & Theron, 1965) and Penicillium (Van Walbeek, Scott & Thatcher, 1968) strains in food production. It has been detected in foodstuffs of both plant and animal origin. OTA is mainly founded as a natural contaminant in cereal products, coffee, cacao, chocolate, spices, raisins, grape juice, wine, beer...(Dall'Asta, Galaverna, Dossena & Marchelli, 2004; Turcotte & Scott, 2011).

OTA is toxic for humans and animals. It has reported to be nephrotoxic, teratogenic, immunosuppressive and has classified as a carcinogen (Fink-Gremmels, Jahn, & Blom, 1995). Due to this toxicity, the governments of many countries have established a maximum residue limits (MRL) for OTA in foods and raw products. The *Codex Alimentarius* Commission (CAC) has adopted an MRL of 5.0 µg/kg for OTA (Walker, 2002).

Consequently, fast and low-cost analytical methods are required for its determination. In foods, there are several methods that have been reported for the determination of OTA including spectrofluorometry (Hult & Gatenbeck, 1976), thin-layer chromatography (TLC), and liquid chromatography (LC) (Engstrom, Richard & Cysewski, 1977). Table 1 gives the comparison between the advantages and disadvantages of these methods and their limits of detection. The most sensitive and selective is actually HPLC-FLD with a limit of detection of 0.05 ng/mL. Time of analysis is short. This automatic analysis is also accurate in the

identification of mycotoxins but the equipment is expensive and need a high level of expertise. ELISA is the technique, which could be compare to aptamer methods. It is also high sensitive, easy to operate, rapid, quite cheap with a low use of organic solvents but this technique could have matrix interference problems and could give false results. Its accuracy is high with a limit of detection around 4 ng/mL. In addition, the cost of aptamers is approximately the same as using ELISA, but aptamer can be regenerated to reuse for several times and that will decrease the cost of research. Even now, many researchers replace the antibodies in ELISA test by an aptamer, which called enzyme-linked apta-sorbent assay (ELASA) (Toh, Citartan, Gopinath, & Tang, 2015; Lee, Zeng, 2017).

Nevertheless, most of these methods could not be applied to coffee and its products without purification because the interfering fluorescent products made the detection very difficult. Regardless of the detection methods used, a sample pretreatment must be performed before the determination of OTA. The methods most frequently used in the extract cleanup are liquid–liquid and solid–liquid extraction like immunoaffinity column, which need to separate the OTA from the matrix components that can interfere by decreasing the sensitivity of the detection analysis and to protect the HPLC column. Therefore, it is still highly desirable to develop simpler and more sensitive methods to detect OTA traces in food samples.

The aptamer development for analytical detection and diagnostic applications has increased in the past few years since first introduced independently by Tuerk & Gold (1990) and Ellington & Szostak (1990). They are short single stranded of artificial oligonucleotides DNA or RNA. They assumes a variety of shapes due to their tendency to form helices and single strand loops. They are able to bind or react with a target molecule with high affinity and high selectivity (Famulok & Mayer, 2014). They are identified in vitro by an iterative selection process called SELEX (Systematic Evolution of Ligands by Exponential enrichment)

(Stoltenburg, Reinemann & Strehlitz, 2007). Aptamers also exhibit excellent stability and wide applicability (Han, Yu & Lee, 2014) which make them suitable for use in medical diagnosis, environmental monitoring and biological analysis (Jeon, Lee, Manjunatha, & Ban, 2013).

Cruz-Aguado & Penner (2008a) have descript an aptamer sequence for the detection of OTA in the elute by immobilizing the aptamer onto a solid phase for the OTA extraction. Then OTA determination was done by liquid chromatography coupled to fluorescence detection. Based on the above-described facts and the report of the first OTA aptamer, a large number of aptamer-based biosensors have been applied to OTA detection., as described by (Hayat, Sassolas, Marty & Radi, 2013; Zhu, 2019; Zeng et al, 2019; Wu, Ma, Zhao, He & Chen, 2018; Yang, Lates, Prieto-Simon, Marty & Yang, 2012; Zhao, Xiang, Chen & Ma, 2019; Zhao, Geng & Wang, 2019).

In this study, we developed an aptamer assisted ultrafiltration cleanup with high performance liquid chromatography-Fluorescence Detector for the determination of OTA in green coffee.

2. Materials and methods

2.1 Materials

Ochratoxin A standard and the specific aptamer for OTA (sequence 5′-GAT-CGG-GTG-TGG-GTG-GCG-TAA-AGG-GAGCAT-CGG-ACA-3′, published by Cruz-Aguado and Penner (Cruz-Aguado & Penner, 2008a) were purchase from Sigma-Aldrich (Saint-Quentin, France). Analytical-grade sodium chloride, potassium chloride were obtained from Carlo Erba (Val de Reuil, France). HEPES (4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid / N-(2-

89 Hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid) and magnesium chloride were purchase
90 from Panreac Química SLU (Barcelona, Spain).

91 The elution buffer, EDTA (ethylenediaminetetraacetic acid) was purchase from Sigma-
92 Aldrich (Saint-Quentin, France), and TRIS (tris hydroxymethyl aminomethane) was purchase
93 from Promega (Auvergne-Rhône-Alpes, France). HPLC-grade methanol was obtained from
94 Carlo Erba (Val de Reuil, France), and acetic acid was purchase from Sigma-Aldrich (Saint-
95 Quentin, France).

96 The HPLC system (Shimadzu LC-10ADVP, Japan) with fluorescence detector (Rf-10AxL
97 and degasser, DGV-14A) consisted of a binary pump (LC Advp), equipped with a reverse-
98 phase analytical column packed with C18 uptisphere type, ODS, 5 µm particle size, 5 ODB,
99 250 x 4.6 mm, with identical pre-column material (Spherisorb 5 µm ODS2, 250 ×4.6 nm).

100 Ultrafiltration Amicon filters, centrifugal filter devices for concentration and purification were
101 purchase from Sigma-Aldrich (Saint-Quentin, France).

102 ### *2.2 **Methods***

103 #### *2.2.1 Preparation of ochratoxin A stock solution and calibration curve*

104 A standard solution 10 µg/mL of ochratoxin A in methanol was used to spike samples and
105 was diluted to obtain working solutions of OTA in methanol for preparing calibration curves.
106 A sept-point calibration curve was done at mass concentrations of working standard solutions
107 of 0.5, 1, 2.5, 5, 10, 25 and 50 ng/mL in methanol/H_2O (3/7). Linear regression using the peak
108 area of the standard as the response versus concentration of OTA has been used to drawn the
109 calibration curve.

110 #### *2.2.2 Preparation of aptamer stock solution*

111 The stock solution of 100 µM aptamer was diluted into two different concentration levels of
112 1 µM and 5 µM in the binding buffer solution (BB) consisting of 10 mM HEPES, 120 mM
113 NaCl, 5 mM KCl, and 5 mM $MgCl_2$ (pH 7) as reported by Cruz-Aguado (Cruz-Aguado &
114 Penner, 2008a). The pH values were adjusted with 2 M NaOH and 2 M HCl.

115 *2.2.3 Preparation of green coffee.*

116 Green coffee species Coffee Arabica origin Nicaragua have been used. Samples were
117 extracted according to the procedure of Cruz-Aguado and Penner (2008) (Cruz-Aguado &
118 Penner, 2008a) slightly modified. In particular, 50 g of green coffee were accurately weighed
119 into an Erlenmeyer flask and extracted with 50 mL methanol/water (60/40, v/v) by orbital
120 shaking for 30 min to have 1 g/mL of extracted green coffee. The extracts of the green coffee
121 were diluted with binding buffer to prepare four different dilutions at concentrations of 0.025,
122 0.05, 0.1, 0.5 g/mL. Then, the extracts were spiked with 15 ng/mL OTA for each dilution.

123 *2.2.4 Aptamer assisted ultrafiltration*

124 The ability of the aptamer-assisted ultrafiltration to bind OTA in standard solution was tested
125 by mixing different concentrations of aptamer with the different extraction solutions of green
126 coffee spiked with 15 ng/mL OTA and incubated for 1 h. These mix were passed through the
127 filter, centrifuge at room temperature at 14000 x g for 30 min and then OTA concentrations
128 were measured in the eluted fractions by HPLC. After that, the OTA bounded to aptamer were
129 eluted with 3 incubations period of 10 min each with the elution buffer (20% methanol in 10
130 mM TRIS and 1 mM EDTA at pH 9) and passed through the same filter to eliminate the
131 bounded OTA and calculate the OTA recovery as described by Cruz-Aguado & Penner
132 (2008a). This experiment was repeated three times.

133 *2.2.5 Quantification of OTA by HPLC-FD*

OTA was quantified by HPLC with a fluorescence detector (Shimadzu RF 20A, Japan). The operating conditions were as follows: injection volume of 100 µL; C18 reverse-phase HPLC column, Uptisphere type, ODS, 5 µm particle size, 5 ODB, 250 x 4.6 mm, with identical pre-column, thermostatically controlled at 35°C; isocratic flow rate of 1mL min-1 (mobile phase: water/acetonitrile/acetic acid, 51:48:1); excitation wavelength of 333 nm and emission wavelength of 460 nm. The contents were calculated from a calibration curve established from an OTA standard (1 µg/mL; ref PD 226 R. Biopharm Rhône Ltd, Glasgow, UK).

2.2.6 Statistical analysis

An experimental design using Doehlert matrix was used to optimize experimental variables and ANOVA (one-way analysis of variance) was used to analyze the data (Doehlert, 1970). The concentration of OTA fixed at 15 ng/mL, the concentration of aptamer and the dilution of green coffee extract were the two variables. Our design consist of one central point and six points forming a regular hexagon. In this experimental design, three aptamer concentrations of 1.47, 4.5 and 7.53 µM were prepared. In addition, five different dilutions of extracted green coffee at concentrations of 0.025, 0.2188, 0.4125, 0.6063 and 0.8 g/mL were prepared.

3. Results and discussion

3.1 Calibration curve of OTA

The calibration curve for the quantification of OTA was constructed using various concentrations. Six calibration points were drawn from six appropriate aliquots of a reference standard, at mass concentrations of working standard solutions 0.5, 1, 2.5, 5, 10, 25 and 50 ng/mL respectively. Volumes of 100 µL of working standard solutions for each aliquots were injected to the HPLC system. The fluorescence of the samples corresponding to the OTA concentration in nanograms, which was calculated by the linear regression equation of the

obtained calibration curve. The calibration curve was constructed by plotting the peak areas (y) obtained from HPLC analysis, versus the concentrations of OTA (x). OTA quantification was reported by comparing peak areas of the samples to the calibration curve of OTA standards. The regression equation was expressed as y = 8E-06x + 0.016, with correlation coefficients R^2= 0.99996.

3.2 *Principles of the Aptamer assisted ultrafiltration method for Ochratoxin A detection*

Ultrafiltration Amicon filter is a centrifugal filter device for concentration and purification of macromolecular components. It consist of polypropylene tube with a vertical regenerated cellulose membrane which working to separate molecules depending of their molecular weight. The molecules with molecular weight within 10 KDa will pass through that filter but the molecules with molecular weight higher than 10 KDa will be blocked in the filter. Knowing that the OTA have a molecular weight of 403.813 g/M, it will pass through that filter. On the other side, the molecular weight of the aptamer conjugated with OTA is more than 11 KDa, and thus will be blocked on the filter (Figure 3). Then, the unbounded OTA concentration were measured in the eluted fractions as described by Cruz-Aguado & Penner (2008a).

3.3 Sample analysis of OTA-aptamers

Cruz-Aguado & Penner (2008a) selected an aptamer that binds OTA with a high affinity that did not bind other compounds structurally similar to OTA. Its affinity for ochratoxin B is 100 times lower than for OTA, whereas these two molecules differ only in the absence of a chlorine atom on ochratoxin B. Many researches have proved the selectivity of this aptamer to Ochratoxin A (OTA) against many other mycotoxins (Lv, Cui, Liang, Quan, Wang & Guo, 2015; Lv, Li, Liu, Cui & Guo, 2017; Lv, Li, Cui, Zhao & Guo, 2017; Zhao, Xiang, Chen &

180 Ma, 2019). This aptamer was used as an oligosorbent for the preparation of aptamer-based
181 SPE columns (Cruz-Aguado & Penner, 2008a; De Girolamo, McKeague, Miller, DeRosa &
182 Visconti, 2011). Starting from preliminary results from Cruz & Penner (2008a) who used this
183 aptamer for binding OTA, we proposed an aptamer assisted ultrafiltration cleanup using
184 HPLC with a Fluorescence detector for the OTA determination. The effect of different
185 parameters, such as aptamer concentration and extraction solutions of green coffee were
186 studied. Different concentration levels (1 µM and 5 µM) of OTA-aptamer and four different
187 extracts of green coffee dilutions at concentration of 0.025, 0.05, 0.1 and 0.5 g/mL, were
188 tested in order to find the most suitable conditions. This experiment was repeated three times.
189 OTA binding percentage to the aptamer assisted ultrafiltration are reported in Figure 4. This
190 process was repeated 3 times.

191 The average percentage of binding depended of the aptamer concentration and the dilutions of
192 extracted green coffee solution. OTA binding and recoveries from the aptamer assisted
193 ultrafiltration are reported in Table 2. Similar binding efficiency towards OTA, total bounded
194 OTA from 42% to 48% were observed in all the extracted dilution of green coffee with
195 aptamer concentration of 1 µM. However, the best binding efficiency was observed in the
196 dilution of 0.025 and 0.05 g/mL of OTA in green coffee with the existence of 5µM aptamer
197 (total OTA binding was 80%). These results showed that the use of aptamer concentration of
198 5 µM was more efficient in the OTA binding process then the concentration of aptamer of 1
199 µM. This was probably due to the higher number of DNA aptamer sites present at the
200 stoichiometry ratio of 1/1 also reported by Cruz-Aguado & Penner (2008b).

201 The percentages of OTA recovery were observed in all the extracted dilution of green coffee
202 and varied from 73.9% to 97.7%, depending on the aptamer concentrations and the dilutions
203 of extracted green coffee. Based on the absence of OTA after the second elution with elution

buffer, the total amounts of recovered OTA, were always less than 100%, it was concluded that about 3% to 15% OTA could be irreversibly retained in the procedure (aptamer and/or Amicon filter).

3.4 Optimization of aptamer assisted ultrafiltration conditions

As reported by Cruz-Aguado & Penner (2008b) specificities and composition of binding buffer solutions played significant roles in improving binding efficiencies. The presence of magnesium as divalent cations is very necessary in the binding process of OTA to the aptamer, due to the formation of a coordination complex between OTA and magnesium linked to both carboxyl and 8-hydroxyl groups in OTA. This complex is enhanced by the highly negatively charged aptamer. At the opposite, the chelating agent EDTA contained in the eluting buffer is necessary to chelate the divalent cations enhancing OTA unbounding from the Aptamer.

According to previous reports, the stability of aptamer depends on the pH due to the decomposition of aptamer in peracidic environment (Kuang et al., 2010). In the same time, OTA is a weak acid and the acidic environment contributed to hold its structural prototypes with pKa values of 4.4 for the carboxylic and 7.5 for the phenolic groups (Monaci & Palmisano, 2004).

The influencing factors and best cleanup conditions were launched on analysis with green coffee samples spiked with fixed concentration of 15 ng/mL OTA. The binding buffer was the same. It consisted of 10 mM HEPES, 120 mM NaCl, 5 mM KCl, and 5 mM $MgCl_2$, and fixed pH 7 as reported by Cruz-Aguado & Penner (2008a). The elution buffer consists of 20% methanol in 10mM TRIS and 1 mM EDTA with pH 9.

Our experimental design consisted of one central point and six points forming a regular hexagon. In this experimental design, the concentration of aptamer and the dilution of green coffee extract were the two variables. Three aptamer concentrations of 1.47, 4.5 and 7.53 µM were prepared. In addition, five different dilutions of extracted green coffee at concentrations of 0.025, 0.2188, 0.4125, 0.6063 and 0.8 g/mL were prepared. The results are reported in Figure 5.

The results showed that the use of 4.5 µM aptamer with 0.025 g/mL of extracted green coffee were optimal in the process of cleaning up OTA in green coffee using aptamer assisted ultrafiltration. The binding percentage of aptamer-OTA increased by increasing the dilution of green coffee extracts. The concentration of 4.5 µM Aptamer, which seemed to be the optimum of binding, was tested with three different dilutions of green coffee extracts, 0.8, 0.4125 and 0.025 g/mL. The binding percentage was observed to be 34%, 65% and 88% respectively. That has showed that the variation of dilution of green coffee extract effect the binding percentage in significant and linear way.

4. Conclusion

The cleanup and analysis of mycotoxins in food is one of the important challenge nowadays. Aptamers are promising materials for this challenge. A simple, rapid, and low cost procedure was described to fabricate aptamer-assisted ultrafiltration for a cleanup and determination of OTA in complex matrices. A simple and rapid extraction method based on methanol/water (60/40, v/v) and purification using aptamer-assisted ultrafiltration was established for the determination of OTA in green coffee. HPLC coupled with a fluorescence detector was performed to detect and quantify OTA. Artificially contaminated green coffee displayed a good range of OTA recoveries up to 97.7%. This method can be applied to the quantitative determination of OTA in green coffee at levels below the maximum levels proposed by the

European Commission for green coffee. This work indicates that aptamer-assisted ultrafiltration methods are useful for the management of OTA in commodities with avoiding complicated experimental setups. It also confirm that aptamers can be used as biorecognition element in diagnostic assays with commercial application for mycotoxin analysis.

In the future, we will evaluate the applicability of that method to the analysis of OTA in other food matrices and contexts in particular directly in container.

Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of this article.

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Figures:

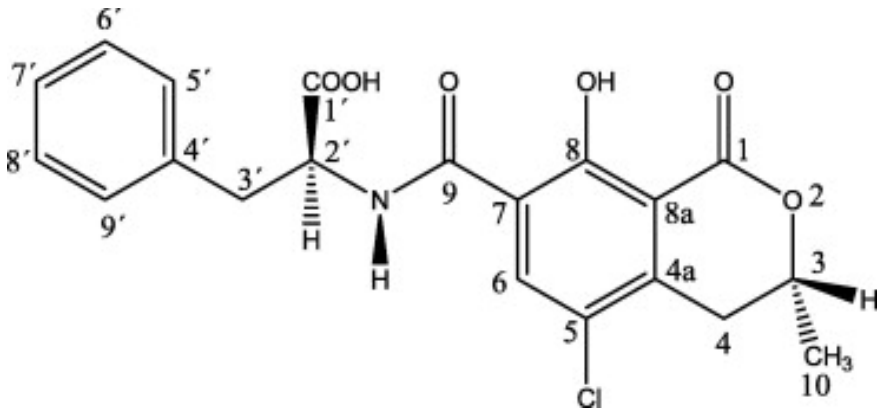

Figure 1: Chemical structure of OTA.

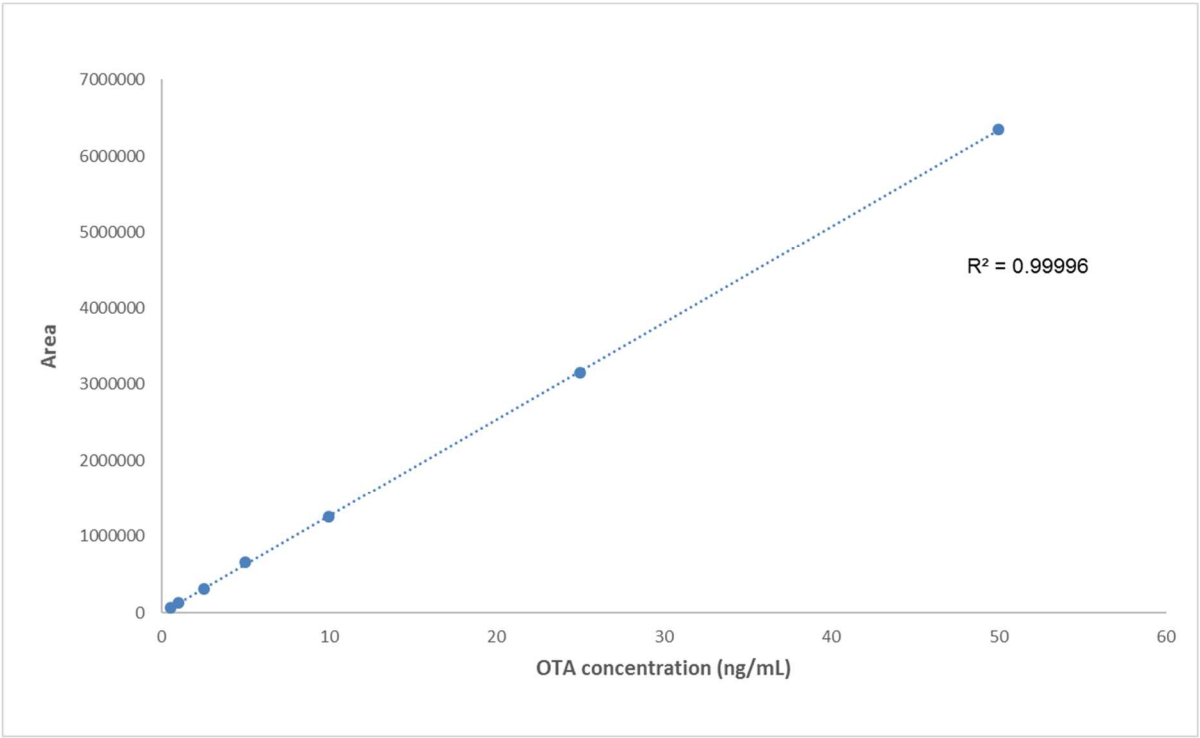

Figure 2: Calibration curve of OTA standards using HPLC-FLD.

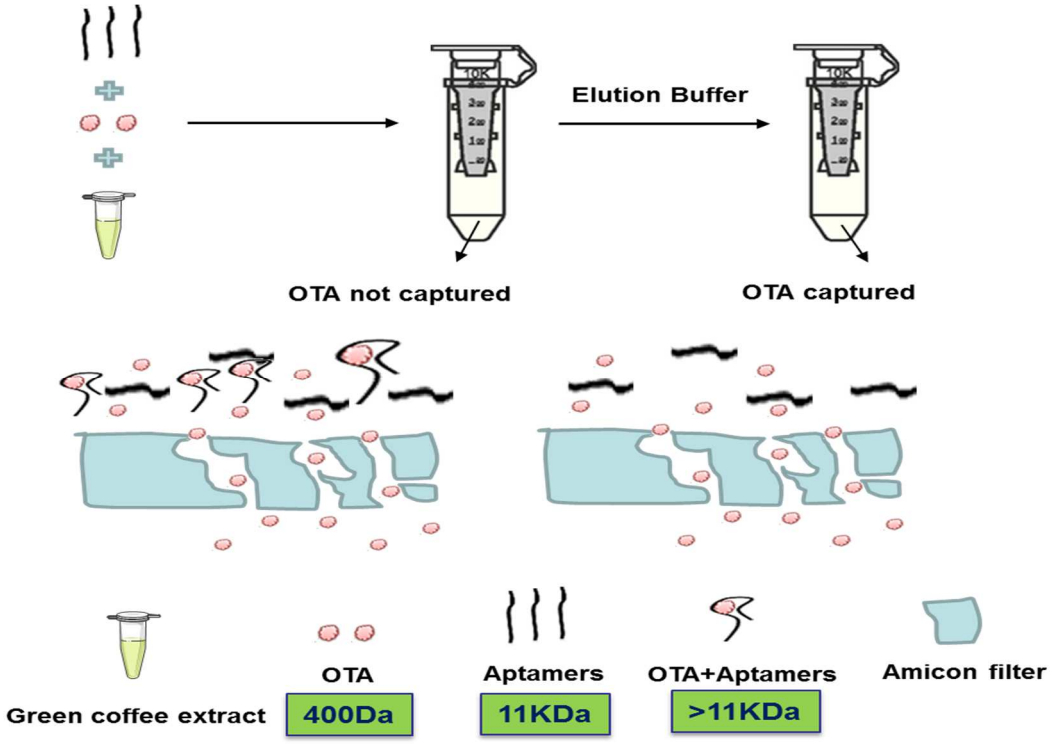

Figure 3: Mechanism of the aptamer assisted ultrafiltration method for OTA detection.

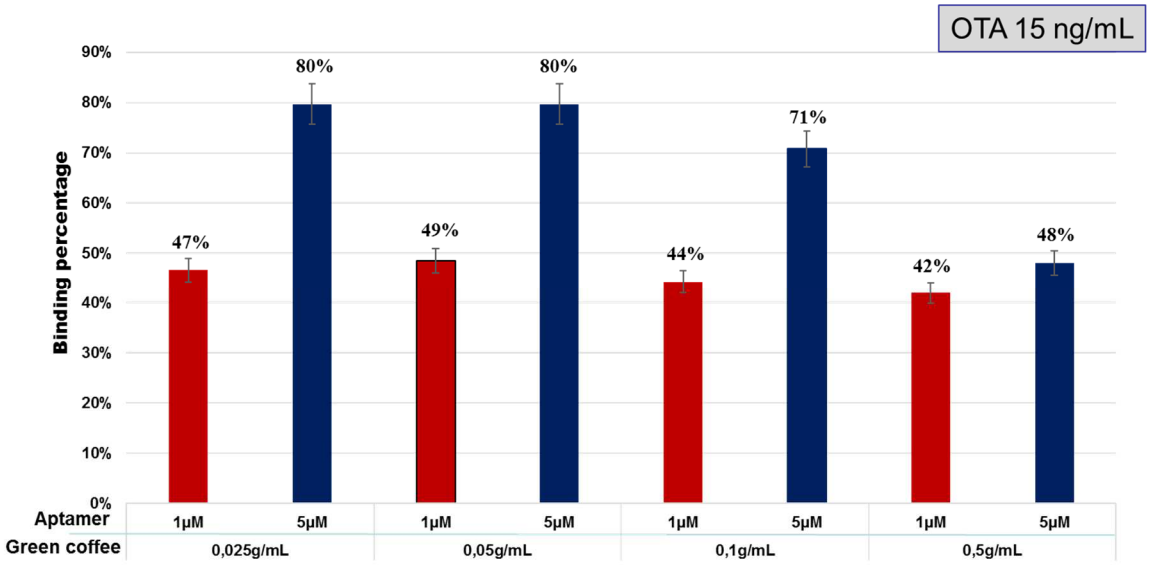

Figure 4: Percentage of OTA captured in different dilutions of green coffee extracts using 5 µM and 1 µM of Aptamers (n=3).

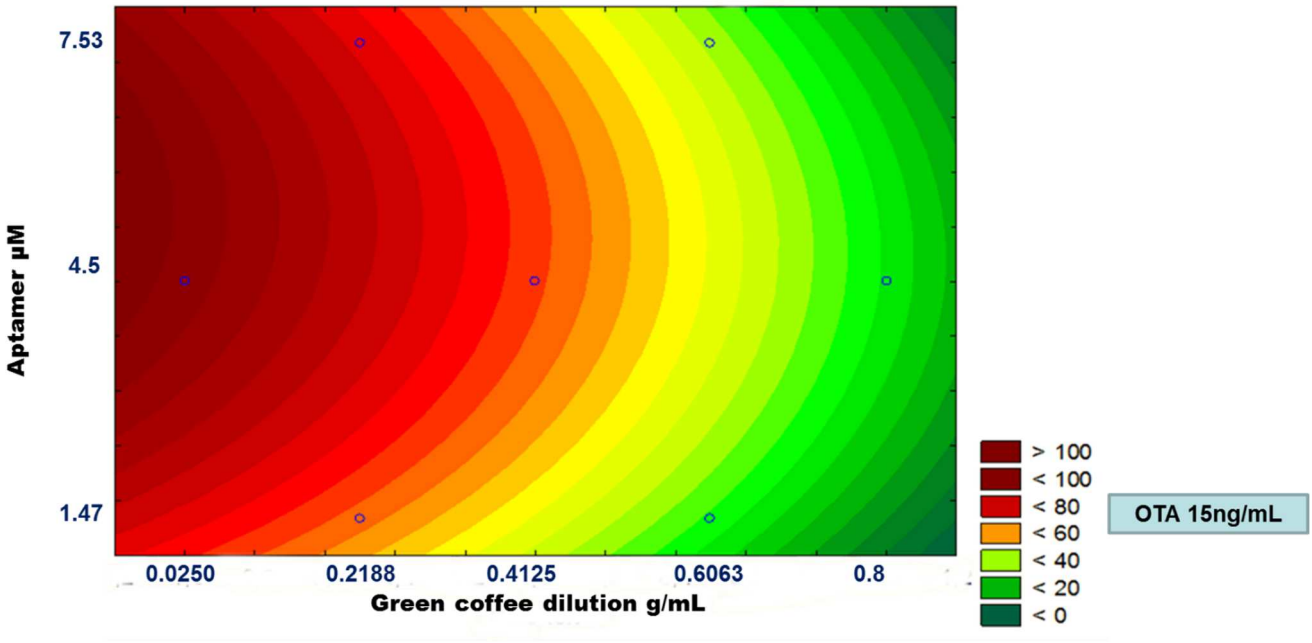

Figure 5: Effects of different green coffee dilutions on the binding percentage of Aptamer-OTA.

Methods	Advantages	Disadvantages	Limit of detection	References
TLC	Simple. Inexpensive.	Low sensitivity. Low precision.	30 ng/mL	(Zhang, Dou, Zhang, Logrieco, & Yang, 2018)
HPLC-FLD	Selectivity. Short analysis time. Automatic analysis. Accurate identification.	Expensive equipment. Expertise required. Derivation required.	0.05 ng/mL	(Kong et al., 2014)
LC-MS	Selective. Sensitive.	Expensive equipment. Expertise required. Sensitivity depends on ionization technique.	2 µg/kg	(Kokkonen, Jestoi, & Rizzo, 2005)
GC	Selective. Sensitive.	Expensive equipment. Expertise required. Derivation required.	0.5 µg/kg	(Turner, Subrahmanyam, & Piletsky, 2009)
ELISA	High sensitivity. Ease of operation. Rapid sample screening. Low use of organic solvents.	Matrix interference problems. Possible false results.	4 ng/mL	(Urusov et al., 2015)
Spectral analysis	Rapid screening. Qualitative and quantitative results.	Spectra overlapping. Complicate interpretation. Possible false results.	100 µg/kg	(Levasseur-Garcia, 2018)

Tables:

Table 1: Comparison between the conventional analytical methods for OTA analysis.

Green coffee extract (g/mL)	Aptamer concentration (µM)	Bounded OTA (% ± SD)	Total OTA recovery (%)
0.025 g/mL	1 µM	46.7 ± 0.53	97.7
	5 µM	79.7 ± 1.65	92.3
0.05 g/mL	1 µM	48.6 ± 0.4	81.9
	5 µM	79.7 ± 2.5	73.9
0.1 g/mL	1 µM	44.3 ± 0.7	88.1
	5 µM	70.8 ± 0.5	75.7
0.5 g/mL	1 µM	42.0 ± 0.8	91.7
	5 µM	48.1 ± 0.4	78.8

Table 2: Percentage of OTA binding on aptamers and OTA recovery from aptamer assisted ultrafiltration (different aptamer concentrations and dilutions of extracted green coffee) (n=3).