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

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28 **Abstract:**

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

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

1 **Abstract**

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

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

19 1. Introduction

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

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

36 Consequently, fast and low-cost analytical methods are required for its determination. In
37 foods, there are several methods that have been reported for the determination of OTA
38 including spectrofluorometry (Hult & Gatenbeck, 1976), thin-layer chromatography (TLC),
39 and liquid chromatography (LC) (Engstrom, Richard & Cysewski, 1977). Table 1 gives the
40 comparison between the advantages and disadvantages of these methods and their limits of
41 detection. The most sensitive and selective is actually HPLC-FLD with a limit of detection of
42 0.05 ng/mL. Time of analysis is short. This automatic analysis is also accurate in the

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

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

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

67 (Stoltenburg, Reinemann & Strehlitz, 2007). Aptamers also exhibit excellent stability and
68 wide applicability (Han, Yu & Lee, 2014) which make them suitable for use in medical
69 diagnosis, environmental monitoring and biological analysis (Jeon, Lee, Manjunatha, & Ban,
70 2013).

71 Cruz-Aguado & Penner (2008a) have described an aptamer sequence for the detection of OTA
72 in the elute by immobilizing the aptamer onto a solid phase for the OTA extraction. Then
73 OTA determination was done by liquid chromatography coupled to fluorescence detection.
74 Based on the above-described facts and the report of the first OTA aptamer, a large number of
75 aptamer-based biosensors have been applied to OTA detection., as described by (Hayat,
76 Sassolas, Marty & Radi, 2013; Zhu, 2019; Zeng et al, 2019; Wu, Ma, Zhao, He & Chen,
77 2018; Yang, Lates, Prieto-Simon, Marty & Yang, 2012; Zhao, Xiang, Chen & Ma, 2019;
78 Zhao, Geng & Wang, 2019).

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

81

82 **2. Materials and methods**

83 ***2.1 Materials***

84 Ochratoxin A standard and the specific aptamer for OTA (sequence 5'-GAT-CGG-GTG-
85 TGG-GTG-GCG-TAA-AGG-GAGCAT-CGG-ACA-3', published by Cruz-Aguado and
86 Penner (Cruz-Aguado & Penner, 2008a) were purchased from Sigma-Aldrich (Saint-Quentin,
87 France). Analytical-grade sodium chloride, potassium chloride were obtained from Carlo Erba
88 (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 mm).

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/H₂O (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 μ M aptamer was diluted into two different concentration levels of
112 1 μ M and 5 μ M in the binding buffer solution (BB) consisting of 10 mM HEPES, 120 mM
113 NaCl, 5 mM KCl, and 5 mM MgCl₂ (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

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

141 *2.2.6 Statistical analysis*

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

149 **3. Results and discussion**

150 *3.1 Calibration curve of OTA*

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

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

162 *3.2 Principles of the Aptamer assisted ultrafiltration method for Ochratoxin A detection*

163 Ultrafiltration Amicon filter is a centrifugal filter device for concentration and purification of
164 macromolecular components. It consist of polypropylene tube with a vertical regenerated
165 cellulose membrane which working to separate molecules depending of their molecular
166 weight. The molecules with molecular weight within 10 KDa will pass through that filter but
167 the molecules with molecular weight higher than 10 KDa will be blocked in the filter.
168 Knowing that the OTA have a molecular weight of 403.813 g/M, it will pass through that
169 filter. On the other side, the molecular weight of the aptamer conjugated with OTA is more
170 than 11 KDa, and thus will be blocked on the filter (Figure 3). Then, the unbounded OTA
171 concentration were measured in the eluted fractions as described by Cruz-Aguado & Penner
172 (2008a).

173 *3.3 Sample analysis of OTA-aptamers*

174 Cruz-Aguado & Penner (2008a) selected an aptamer that binds OTA with a high affinity that
175 did not bind other compounds structurally similar to OTA. Its affinity for ochratoxin B is 100
176 times lower than for OTA, whereas these two molecules differ only in the absence of a
177 chlorine atom on ochratoxin B. Many researches have proved the selectivity of this aptamer to
178 Ochratoxin A (OTA) against many other mycotoxins (Lv, Cui, Liang, Quan, Wang & Guo,
179 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 5 μM 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

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

207 *3.4 Optimization of aptamer assisted ultrafiltration conditions*

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

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

221 The influencing factors and best cleanup conditions were launched on analysis with green
222 coffee samples spiked with fixed concentration of 15 ng/mL OTA. The binding buffer was the
223 same. It consisted of 10 mM HEPES, 120 mM NaCl, 5 mM KCl, and 5 mM MgCl₂ , and
224 fixed pH 7 as reported by Cruz-Aguado & Penner (2008a). The elution buffer consists of
225 20% methanol in 10mM TRIS and 1 mM EDTA with pH 9.

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

232 The results showed that the use of 4.5 μM aptamer with 0.025 g/mL of extracted green coffee
233 were optimal in the process of cleaning up OTA in green coffee using aptamer assisted
234 ultrafiltration. The binding percentage of aptamer-OTA increased by increasing the dilution of
235 green coffee extracts. The concentration of 4.5 μM Aptamer, which seemed to be the
236 optimum of binding, was tested with three different dilutions of green coffee extracts, 0.8,
237 0.4125 and 0.025 g/mL. The binding percentage was observed to be 34%, 65% and 88%
238 respectively. That has showed that the variation of dilution of green coffee extract effect the
239 binding percentage in significant and linear way.

240 **4. Conclusion**

241 The cleanup and analysis of mycotoxins in food is one of the important challenge nowadays.
242 Aptamers are promising materials for this challenge. A simple, rapid, and low cost procedure
243 was described to fabricate aptamer-assisted ultrafiltration for a cleanup and determination of
244 OTA in complex matrices. A simple and rapid extraction method based on methanol/water
245 (60/40, v/v) and purification using aptamer-assisted ultrafiltration was established for the
246 determination of OTA in green coffee. HPLC coupled with a fluorescence detector was
247 performed to detect and quantify OTA. Artificially contaminated green coffee displayed a
248 good range of OTA recoveries up to 97.7%. This method can be applied to the quantitative
249 determination of OTA in green coffee at levels below the maximum levels proposed by the

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

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

256 **Declaration of interest**

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

259

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Figures:

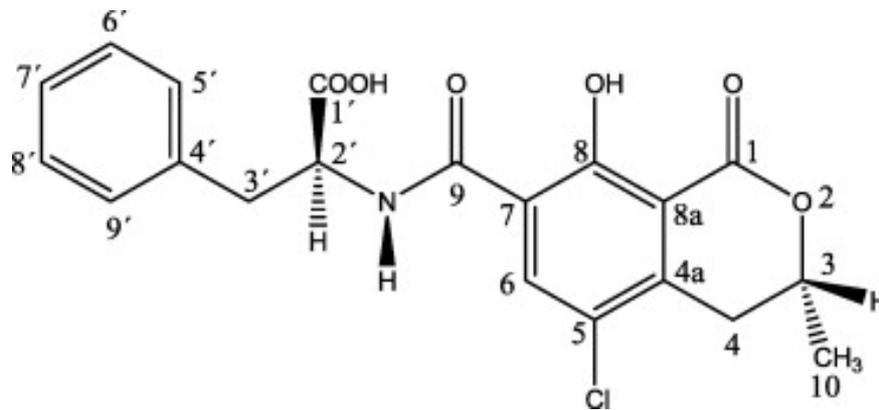


Figure 1: Chemical structure of OTA.

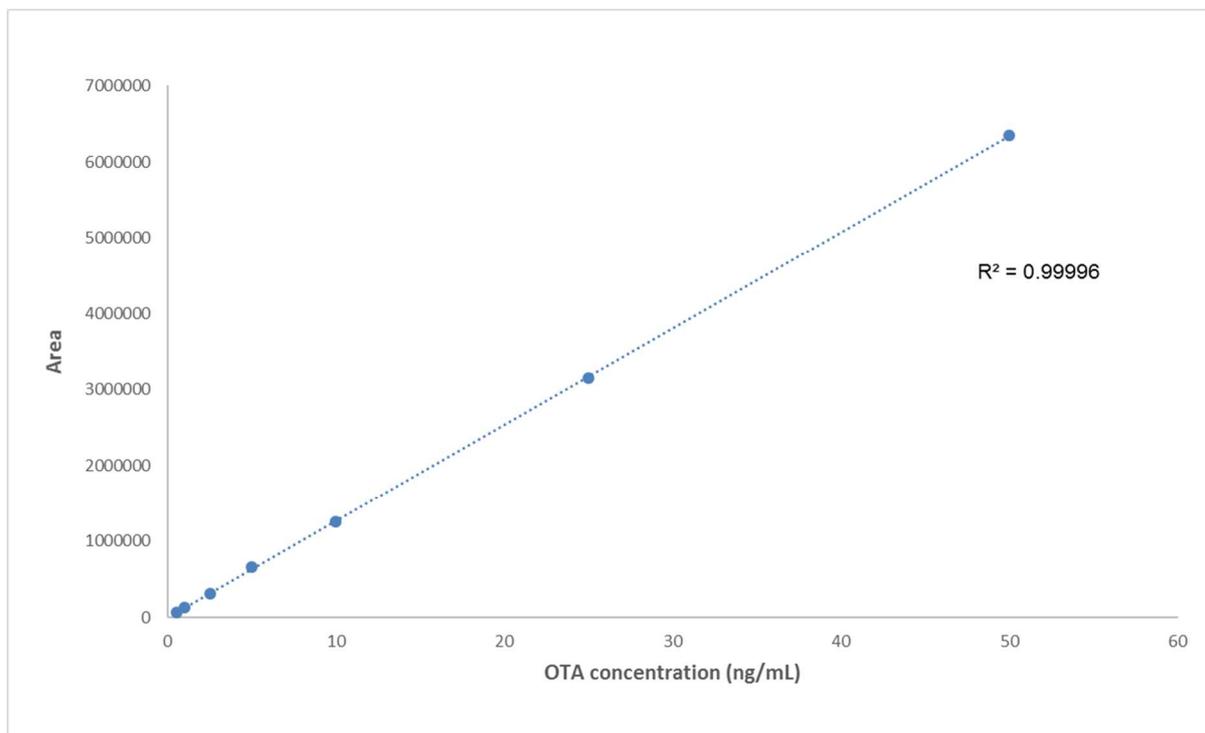


Figure 2: Calibration curve of OTA standards using HPLC-FLD.

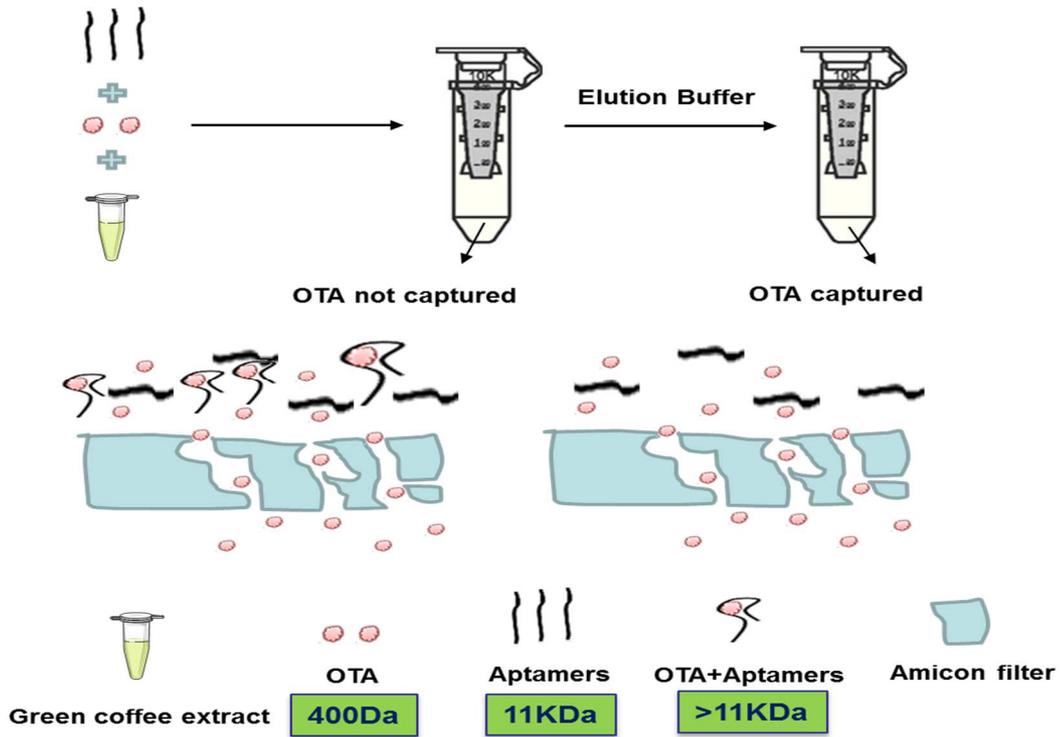


Figure 3: Mechanism of the aptamer assisted ultrafiltration method for OTA detection.

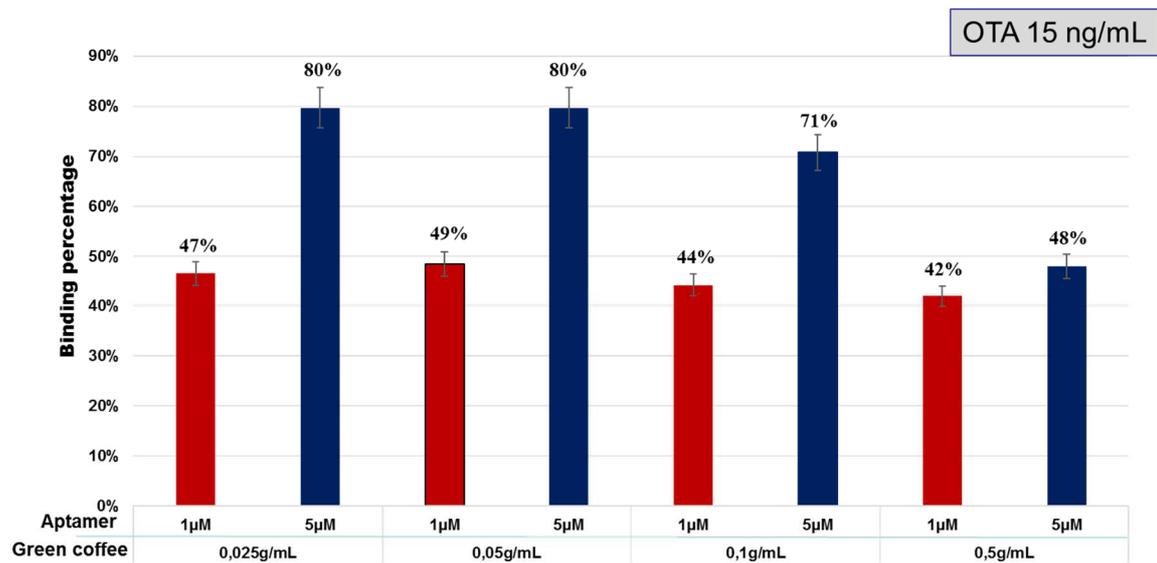


Figure 4: Percentage of OTA captured in different dilutions of green coffee extracts using 5 μM and 1 μM of Aptamers (n=3).

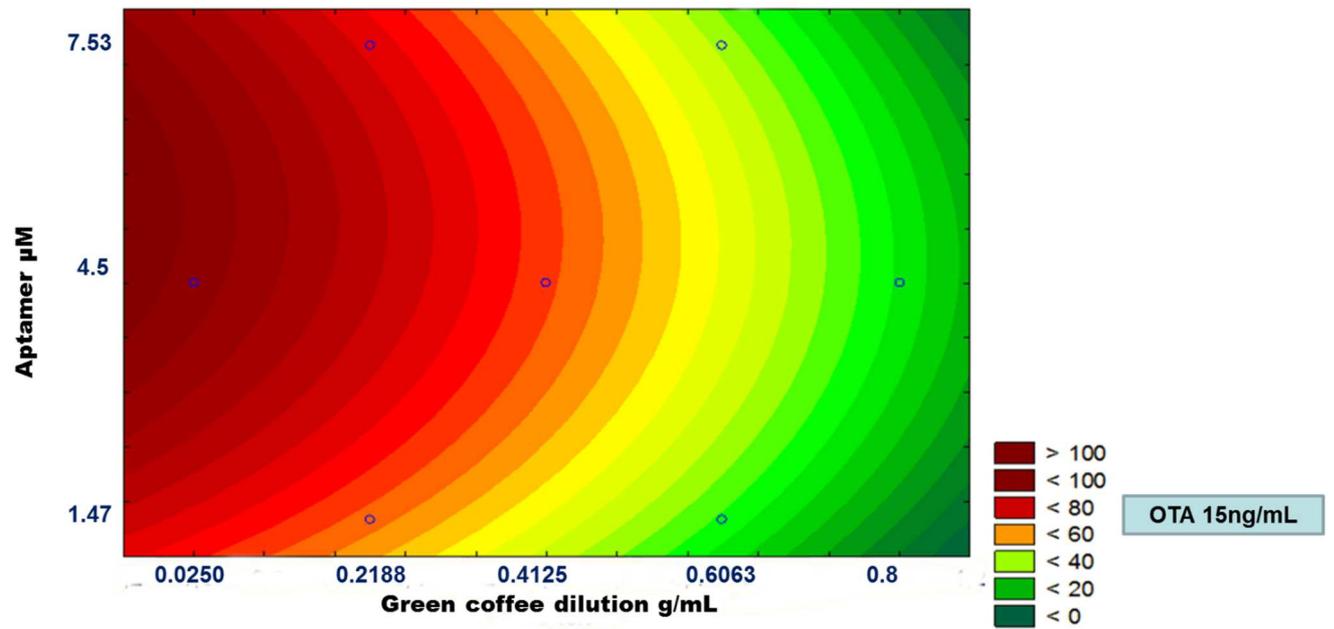


Figure 5: Effects of different green coffee dilutions on the binding percentage of Aptamer-OTA.

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 ($\% \pm \text{SD}$)	Total OTA recovery (%)
0.025 g/mL	1 μM	46.7 \pm 0.53	97.7
	5 μM	79.7 \pm 1.65	92.3
0.05 g/mL	1 μM	48.6 \pm 0.4	81.9
	5 μM	79.7 \pm 2.5	73.9
0.1 g/mL	1 μM	44.3 \pm 0.7	88.1
	5 μM	70.8 \pm 0.5	75.7
0.5 g/mL	1 μM	42.0 \pm 0.8	91.7
	5 μM	48.1 \pm 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).