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1 **Development of the custom polymeric materials specific for**
2 **aflatoxin B1 and ochratoxin A for application with the**
3 **ToxiQuant T1 sensor tool**

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8
9 **ABSTRACT**

10 Two polymers were computationally designed with affinity to two of the most
11 abundant mycotoxins aflatoxin B1 (AFB1) and ochratoxin A (OTA) for application in
12 the ToxiQuant T1 System. The principle of quantification of AFB1 and OTA using
13 the ToxiQuant T1 instrument comprised of a fluorimetric analysis of mycotoxins
14 adsorbed on the polymer upon exposure to UV light. High affinity of the developed
15 resins allowed the adsorption of both toxins as discrete bands on the top of the
16 cartridge with detection limit as low as 1 ng quantity of mycotoxins.

17 *Keywords: Mycotoxins, aflatoxin B1, ochratoxin A, computational modelling, solid-*
18 *phase extraction.*

19
20 **1. Introduction**

21 The name mycotoxin is a combination of the Greek word for fungus “mykes” and the
22 Latin word “toxicum” meaning poison. The term “mycotoxins” is reserved for the
23 toxic chemical products which are mainly produced by five fungal genera *Aspergillus*,
24 *Penicillium*, *Fusarium*, *Alternaria* and *Claviceps* (1) that readily colonise crops either
25 in the field or after harvest. These compounds pose a potential threat to human and
26 animal health, through the ingestion of food products prepared from these
27 commodities. Each mycotoxin is produced by one or more specific fungal species.

28 Among the most significant mycotoxins are aflatoxins, a group of toxins produced by
29 the fungi *Aspergillus flavus* and *A. parasiticus* and ochratoxins, a group produced by
30 some species of *Aspergillus* (*A. ochraceus*) and by *Penicillium verrucosum*.

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33

34 Aflatoxins, particularly AFB₁, have received great attention due to their acute
35 toxicological effects in humans. The International Agency for Research and Cancer
36 (IARC) included AFB₁ as a primary group of carcinogenic compounds (2). Many
37 countries have strict regulatory limits on commodities intended for human and animal
38 consumption. The legal limits for aflatoxins for human consumption are 0-50 ng g⁻¹
39 (3) and for animal feed are 1-20 µg g⁻¹ (4). Ochratoxin A (OTA) is a weak organic
40 acid which is also classified by IARC as a compound which is carcinogenic to
41 humans and animals (2). According to European Commission regulations the
42 maximum limit OTA in food should be restricted to 5 or 10 µg l⁻¹ (roasted and instant
43 coffee correspondingly) and 2 µg l⁻¹ (grape juice and wine) (5).

44 Since the mere presence of *Aspergillus* or *Penicillium* does not always mean
45 the presence of toxins in the substrate the determination of toxins in the food samples
46 is essential. The main methods for detection of aflatoxins and ochratoxins in food
47 which are high performance liquid chromatography (HPLC) equipped with
48 immunoaffinity columns and fluorescent detectors and thin layer chromatography
49 (TLC) (6, 7). The lowest level of aflatoxin quantification using HPLC method is 0.1
50 ng g⁻¹. For quantitative testing of multiple samples radioimmunoassay and enzyme-
51 linked immunosorbent assays (ELISA) are also used. According to Association of
52 Official Analyst Chemists International (AOAC International) the detection limit of
53 AFB₁ using ELISA method is 9-20 ng g⁻¹ (7). Despite sufficient sensitivity and high
54 analytical efficiency HPLC quantification requires expensive equipment and highly
55 trained personnel. Although ELISA could be considered as a sensitive and economical
56 solution, it is difficult to perform it in the “point- of- care” situation where very rapid
57 but affordable tests are mandatory. Market research shows that there is a high demand
58 for a simple, rapid and affordable testing tool in order to provide a simple and
59 quantitative analysis of the food for presence of toxins throughout the global food
60 chain. The ToxiQuant T1 system has been developed to meet the demands of this
61 niche of diagnostic market (Toximet Ltd. UK) (Fig. 1).

62 The ToxiQuant T1 instrument consists of a UV light source, an automatically
63 adjustable cartridge holder, a detector, necessary optics, mechanics and software. The
64 samples are loaded onto cartridges using standard SPE manifold equipped with a
65 vacuum pump or manually, using a syringe. The mobile phase is filtered through the
66 adsorbent which was designed to adsorb the analytes of interest (particularly

67 fluorescent mycotoxins) as a band on the top of the packed resin. The ToxiQuant T1
68 instrument scans the semi-transparent plastic cartridge under conditions which
69 stimulate the fluorescence of mycotoxin adsorbed on the top of the polymeric resin
70 and provides a quantitative measurement of the concentration of toxin present in the
71 sample (Fig. 2). The requirements for the polymeric material are high affinity towards
72 AFB1 and OTA and low background fluorescence.

73

74 **2. Experimental**

75 *2.1 Chemicals and materials*

76 AFB1, OTA and deoxynivalenol (DON) were obtained from Sigma (Sigma-Aldrich,
77 UK). N,N'-methylene bisacrylamide (MBAA), diethylaminoethylmethacrylate
78 (DEAEM), itaconic acid (IA) ethylene glycol dimethacrylate (EGDMA) and 1,1-
79 azobis (cyclohexanecarbonitrile) were purchased from Aldrich (Sigma-Aldrich, UK).
80 Dimethylformamide (DMF), HPLC grade methanol and HPLC grade water were
81 obtained from Acros (Fisher Scientific, UK). 1-ml empty SPE cartridges were
82 purchased from Supelco (Sigma-Aldrich, UK).

83

84 *2.2 Computational modelling*

85 The molecular modelling was made using a workstation from Research
86 Machines running the CentOS 5 GNU/Linux operating system. The workstation was
87 configured with a 3.2GHz core 2 duo processor, 4 GB memory and a 350 GB fixed
88 drive. This system was used to execute the software packages SYBYL 7.0TM (Tripos
89 Inc., St. Louis, Missouri, USA). The LEAPFROG algorithm was used to screen the
90 library of functional monomers for their possible interactions with the template
91 resulting in a table ranking the monomers with the highest binding score (kcal mol^{-1})
92 as the best candidates for polymer preparation. The library contained 21 functional
93 monomers commonly used in molecular imprinting which possess polymerisable
94 residues and residues able to interact with a template through ionic and hydrogen
95 bonds, van der Waals' and dipole-dipole interactions (8). [The more detailed](#)
96 [description of the molecular modelling protocol and functional monomers library](#)
97 [\(Fig. 1S\) is included in the Supplementary Information chapter.](#) Energy minimisation
98 was performed on each of the monomers in the database to a value of $0.001 \text{ kcal mol}^{-1}$.
99 The screening was conducted "in the vacuum" which was determined by carrying
100 the simulation at dielectric constant equal 1. The goal of the screening was to select

101 the functional monomer which has sufficient binding energy towards the toxin. The
102 result of the modelling was a virtual prediction of a molecular complex which could
103 be formed between the toxin and selected functional monomer. Usually, several
104 functional monomers were selected for polymer preparation and a choice of the best
105 one was determined by laboratory testing under conditions which will be required for
106 the practical application.”

107

108 *2.3 Polymers preparation*

109 The polymers were prepared from methacrylate functional monomers and
110 cross-linker by free-radical polymerisation approach (9). Based on the computational
111 modelling, MBAA was selected for the preparation of the polymer specific for AFB1
112 (P1) and a mixture of DEAEM and IA was selected for preparation of polymer
113 specific for OTA (P2). P1 composition: 10 g of the polymer with 0.5 g of MBAA, 9.5
114 g of cross-linker (EGDMA) and 100 mg of 1,1-azobis (cyclohexanecarbonitrile) as an
115 initiator. P2 composition: 1 g of IA with 1g of DEAEM, 8 g of EGDMA and 100 mg
116 of 1,1-azobis (cyclohexanecarbonitrile) as initiator. 10 g of dimethylformamide was
117 used in both cases as a porogen. Polymers were polymerised on ice at +4 °C using
118 Cermax UV lamp (PerkinElmer, UK). After synthesis both polymers were ground and
119 sieved using Ultracentrifuge Mill and Shaker (Retsch, UK). Fractions with size
120 particles size 25-63 µm, 63-125 µm and 125-200 µm were collected. The polymer
121 were thoroughly washed with methanol using Soxhlet extraction, dried and used for
122 SPE.

123

124 *2.4 SPE protocol and regeneration of polymers*

125 75 mg of the polymers (P1 or P2) were packed in the 1-ml SPE cartridges and
126 were conditioned with 2 ml of HPLC-grade water. 1 ml of 80% methanol which was
127 used for extraction of aflatoxin was spiked with 1-200 ng of AFB1. Before the
128 loading the spiked extraction solution was diluted 4 times with water and loaded into
129 the cartridge. For analysis of OTA adsorption the extraction solution, which consisted
130 of 60% aqueous acetonitrile, was spiked with 1- 300 ng of OTA. Before the loading
131 the spiked extraction solution was diluted 4 times with water to decrease the
132 acetonitrile content to 15% and 4 ml were filtered through the cartridges packed with
133 P2 polymer. 1 ml of 15% acetonitrile (OTA) or 20% methanol (AFB1) was used to
134 wash the cartridges before the measurement using the ToxiQuant-T1 instrument. The

135 protocols for regeneration of the P1 and P2 polymers were optimised. In order to
136 regenerate the P1 polymer it was washed with 4 ml of methanol followed by 4 ml of
137 water. For regeneration of the P2 polymer the following washing steps were
138 conducted: 2 ml of water, 4 ml of 50% methanol containing 0.1 M NaOH, 4 ml of
139 water, 4 ml of 0.1 M HCl, 4 ml of methanol, 4 ml of water. These treatments were
140 sufficient for complete regeneration of the polymers and preparation for the next
141 loading experiment.

142

143 **3. Results and discussion**

144

145 *3.1 Computational modelling*

146 Molecular structures of AFB1 and OTA were drawn, minimised and screened
147 against a virtual library of the functional monomers using the LEAPFROG algorithm
148 resulting in tables ranking the monomers with the highest binding score (Suppl.
149 Inform., Table 1S). It was found that MBAA is a functional monomer which could
150 provide a high binding towards AFB1 (binding energy- $-32.26 \text{ kcal mol}^{-1}$). This
151 monomer forms two hydrogen bonds with two oxygens of the furan and coumarin
152 rings of the molecule of AFB1 (Fig. 3). Among other polymers which were also
153 screened for aflatoxin adsorption were polymers based on acrylamide, allylamine,
154 ethylene glycol methacrylate phosphate (EGMP) and methacrylic acid. MBAA-based
155 polymer demonstrated superior adsorption properties towards AFB1 and was selected
156 for the future experiments.

157 The screening of OTA against the virtual library of functional monomers showed that
158 charged DEAEM demonstrated the highest binding towards OTA (binding energy: -
159 $61.10 \text{ kcal mol}^{-1}$). Hydrogen bonds were formed between the charged amino group of
160 DEAEM and carboxyl group of the phenylalanine moiety of ochratoxin A (Fig. 4).
161 Since the pKa of the carboxylic group of OTA is 4.4 (10), it means that OTA in the
162 neutral loading solution is negatively charged. In order to induce the positive charge
163 of the DEAEM monomer and to increase the OTA binding, a second functional
164 monomer, IA (binding energy: $-26.74 \text{ kcal mol}^{-1}$) was selected for the polymer
165 preparation. Molecular modelling showed that IA formed bonds with hydroxyl and
166 carbonyl groups of the benzopyran moiety of OTA (Fig. 4). Based on the results of
167 molecular modelling IA and DEAEM were selected for polymer preparation.

168 Among other polymers which were screened for OTA adsorption were
169 polymers based on acrylamide, methylene bisacrylamide (MBAA), 4-vinyl pyridine
170 (4-VP) and IA. Since polymer based on the combination of DEAEM and IA
171 demonstrated superior adsorption properties towards OTA, it was used for the future
172 experiments.

173

174 *3.2 Polymers testing*

175 The P1 and P2 polymers were prepared as described in the Experimental
176 section. During the polymer's preparation special efforts were made to comply with
177 requirements of the ToxiQuant T1 instrument and produce polymers with a low
178 background reading. Although cross-linked methacrylate polymers do not have
179 intrinsic fluorescent properties, some light scattering occurs when they are scanned
180 using the ToxiQuant T1 instrument. In order to reduce the background signal,
181 polymers were prepared using UV polymerisation at low temperature (below +4 ° C).
182 Low-temperature polymers demonstrated significantly lower background value when
183 used in the ToxiQuant T1 instrument than polymers prepared by thermo-
184 polymerisation. This observation could be explained by the lower polymerisation rate,
185 at low temperature, which resulted in more regular homogeneous gel-like morphology
186 polymers (11). Also selection of the particle size with the lowest background reading
187 was carried out. Several fractions with different sizes were tested. Fraction 63-125 µm
188 demonstrated the lowest background reading and was selected for future work.

189 The composition of the loading solution was also optimised. It was found that
190 although P1 and P2 polymers could adsorb the corresponding toxins directly from the
191 extraction solution (80% methanol for AFB1 and 60% of acetonitrile for OTA) the
192 peaks were too wide and could not be used for quantification using the ToxiQuant T1
193 instrument. Four-time dilution of the extraction sample resulting in 20% methanol
194 content for the loading of AFB1 and 15% acetonitrile for OTA loading was
195 considered as optimal. This ensured that the toxin band was situated on the top of the
196 polymer and generated a strong signal when scanned by the ToxiQuant T1 instrument.

197 In order to test the polymers for adsorption of AFB1, 75 mg of P1 polymer
198 (fraction 63-125 µm) was packed into 1-ml SPE tubes and conditioned with 2 ml of
199 HPLC grade water using a vacuum manifold. The optical absorbance of the pre-
200 conditioned cartridges was measured and used as a background value for the
201 quantification of AFB1 in the samples. 4-ml aliquots of 20% methanol containing

202 different concentrations of AFB1 were loaded into SPE cartridges and measured using
203 the ToxiQuant T1 instrument. A calibration curve was made by plotting the height
204 values of the peaks measured by the ToxiQuant T1 instrument for different amounts
205 of AFB1 obtained after subtraction of the baseline value (Fig. 5). This calibration was
206 linear in the range between 10 and 200 ng of AFB1.

207 In order to assess the possibility of pre-concentrating the samples and assess
208 the limit of detection using the ToxiQuant instrument, 10 ml of 20% methanol were
209 spiked with 1 ng of AFB1 and loaded onto P1 polymer. A photograph of the control
210 cartridge which did not contain toxin and a cartridge loaded with 1 ng of AFB1 was
211 taken under UV light using a transilluminator Gene Genius Bio Imaging system
212 (Synoptics Ltd, USA). It is possible to see a band of 1 ng of AFB1 which is adsorbed
213 by P1 polymer (Fig. 6). Based on the observation that the aflatoxin was adsorbed on
214 the top of the polymer it is possible to assume that the polymer has high affinity
215 towards aflatoxin and could be used in combination with the ToxiQuant instrument
216 platform. The minimal limit of detection was estimated as 1 ng of AFB1.

217 In order to test the polymers for adsorption of OTA, 75 mg of the P2 polymer
218 (fraction 63-125 μm) was packed into 1-ml SPE tubes and pre-conditioned with 2 ml
219 of HPLC-grade water on the vacuum manifold. 4-ml aliquots of 15% acetonitrile were
220 spiked with different amounts of OTA and loaded onto SPE cartridges and measured
221 using the ToxiQuant instrument. A calibration curve was plotted using the height of
222 the peaks for different concentration of OTA after subtraction of the baseline value.
223 This calibration was linear in the range between 10 and 300 ng of OTA (Fig. 7).

224 It was shown that the P2 polymer is able to adsorb 1 ng of OTA from 10 ml of
225 diluted solution (Fig. 8). It also shows that the P2 polymer has sufficient affinity
226 towards OTA in the wide range of concentrations. The limit of quantification of OTA
227 (S/N=10) was under 1 ng.

228 Since the detection of ToxiQuant T1 sensor is based on the measurement of
229 fluorescent compounds, it was expected that non-fluorescent toxins or other
230 contaminants could not affect the quantification of AFB1 or OTA using ToxiQuant
231 T1 sensor. The cross-reactivity of the developed polymers was tested with toxin
232 deoxynivalenol (DON) under conditions similar to AFB1 and OTA adsorption. It was
233 found that DON was not adsorbed by either P1 or P2 polymer.”

234 The protocol for washing and regeneration of both polymers was optimised. It
235 was found that it was possible to regenerate and to re-use the cartridges up to 10 times
236 without losing the polymer's binding capacity to their respective targets.

237

238 **4. Conclusions**

239 Two rationally-designed polymers were developed for adsorption of aflatoxin B1 and
240 ochratoxin A for the application with the ToxiQuant T1 instrument. It was found that
241 both polymers have high affinity towards their corresponding toxins and could be
242 used for detection of 1 ng of the toxins. Since the principle of operation of the
243 ToxiQuant instrument did not require the elution of the toxin from the SPE column, it
244 minimised the danger of contamination when using the toxin and decreases the time
245 of the analysis. Future study will be directed towards the validation of the ToxiQuant
246 software for quantification of mycotoxins and for application of the developed
247 polymers for extraction of the AFB1 and OTA from complex food matrices.

248 It is important to emphasise that the ToxiQuant T1 System, in combination with
249 designer resins which are custom-produced for specific analytes, could be used for a
250 broad range of applications which require an inexpensive and rapid quantification of
251 the compound of interest, such as environmental pollutants, drugs of abuse and
252 counterfeit drugs.

253

254 **Acknowledgement**

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256

257 **References**

- 258 [1] P. S. Steyn, *Toxicol. Lett.* 82/83 (1995) 843.
259 [2] International Agency for Research on Cancer (IARC), 1993. IARC Monograph
260 Evaluation of Carcinogenic Risk to Humans No. 56.
261 [3] J. Stroka, E. Anklam, *Trends Anal. Chem.* 21 (2002) 90.
262 [4] Food and Agriculture Organisation of the United Nations. Worldwide regulations
263 for mycotoxins in food and feed in 2003. FAO Corporate Document Repository,
264 Rome 2004.
265 [5] Commission Regulation (EC) No. 123/2005 of 26 January 2005, Official Journal
266 of the European Union. L25 (2005) 3.
267 [6] M. J. O' Riordan, M.G. Wilkinson, *Food Chem.* 107 (2008) 1429.

- 268 [7] AOAC, Official methods of Analysis (15th ed.), Natural toxins, method number
269 991.31 Vol. II, AOAC (1995).
- 270 [8] S. A. Piletsky, K. Karim, E. V. Piletska, C. J. Day, K. W. Freebairn, C. Legge, A.
271 P. F. Turner, Analyst 126 (2001) 1826.
- 272 [9] P. A. G. Cormack, A. Z. Elorza, J. Chromat. B 804 (2004) 173.
- 273 [10] H. Valenta, J. Chromatogr. A 815 (1998) 75.
- 274 [11] S. A. Piletsky, E. V. Piletska, K. Karim, K. W. Freebairn, C. H. Legge, A. P. F.
275 Turner, Macromolecules, 35 (2002) 7499.

276

277 **Figure captions:**

278 Figure 1. The ToxiQuant T1 prototype instrument

279 Figure 2. The principle of toxin quantification using the ToxiQuantT1 sensor.

280 Figure 3. 3-D molecular complex of AFB1 with MBAA functional monomer (top
281 picture; oxygen atoms are shown in red, carbon atoms are white and the light blue
282 atoms are hydrogen) and 2-D structure of aflatoxin B1 (bottom picture).

283 Figure 4. Molecular complex between negatively charged OTA and functional
284 monomers DEAEM and IA (top picture; oxygen atoms are shown in red, carbon
285 atoms are white and the light blue atoms are hydrogen) and 2-D structure of OTA
286 (bottom picture).

287 Figure 5. Calibration curve for quantification of AFB1 using the ToxiQuant T1
288 instrument.

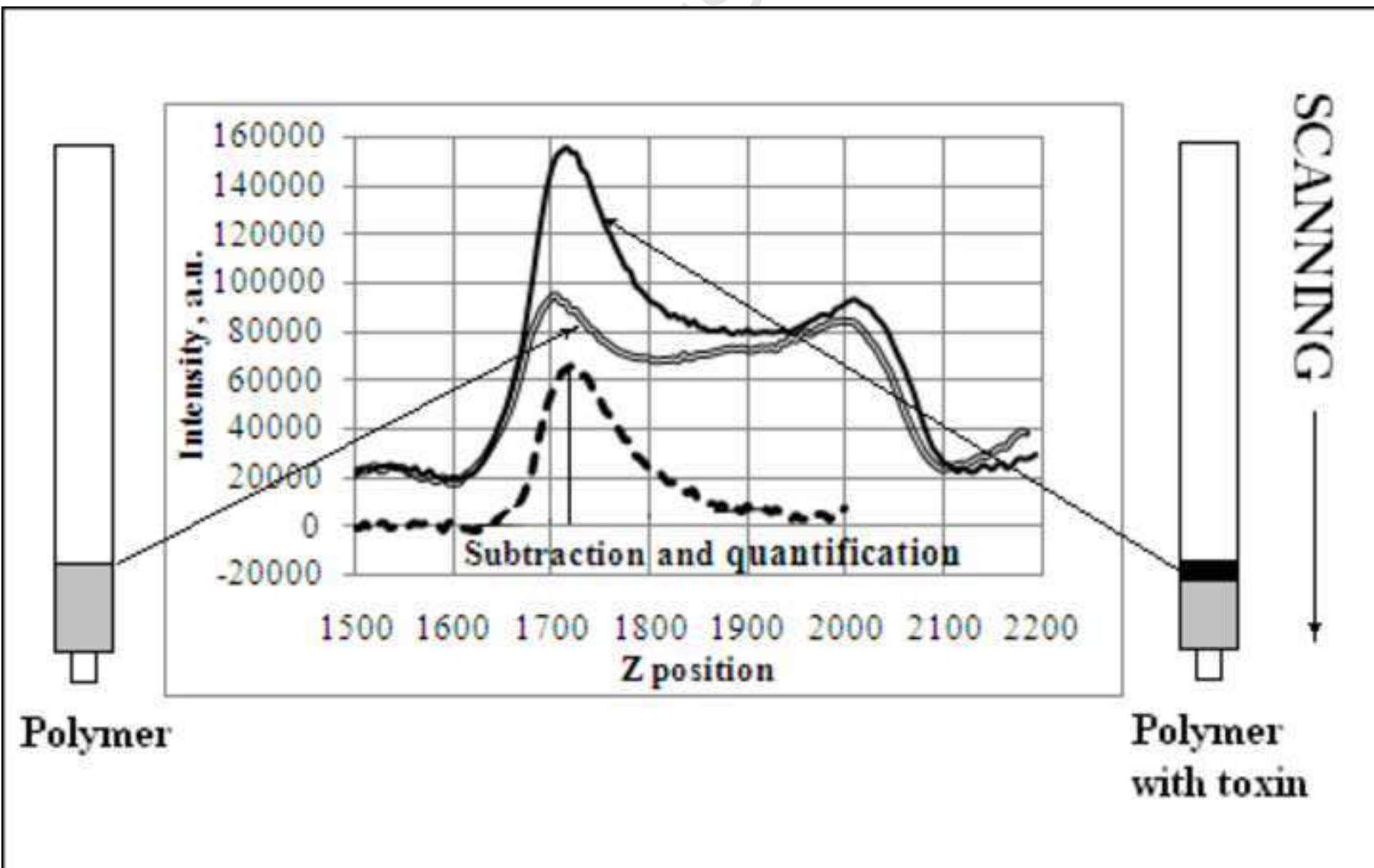
289 Figure 6. P1 polymer with adsorbed 1 ng of AFB1 (on the right), on the left- control
290 cartridge without toxin.

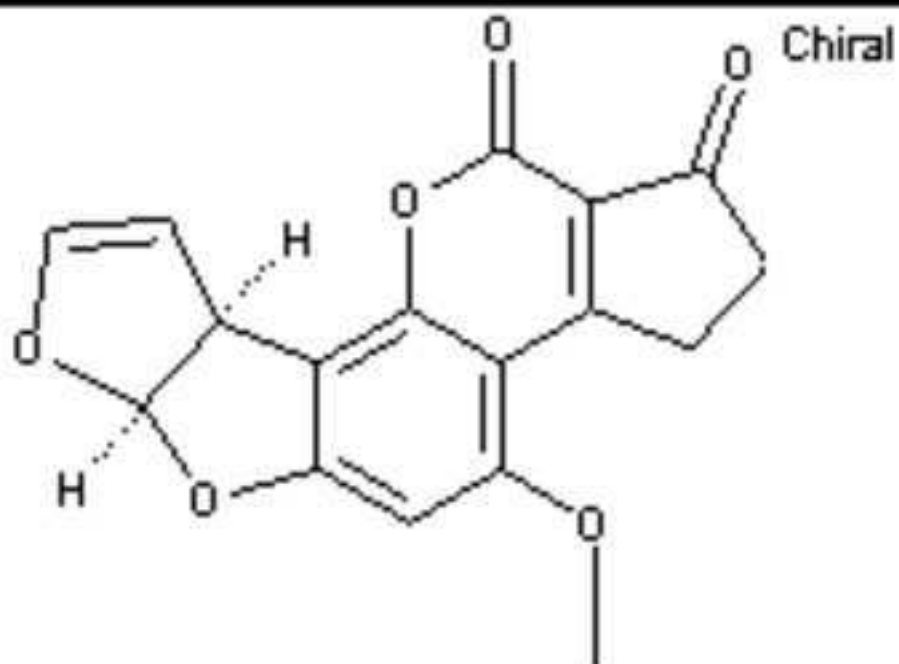
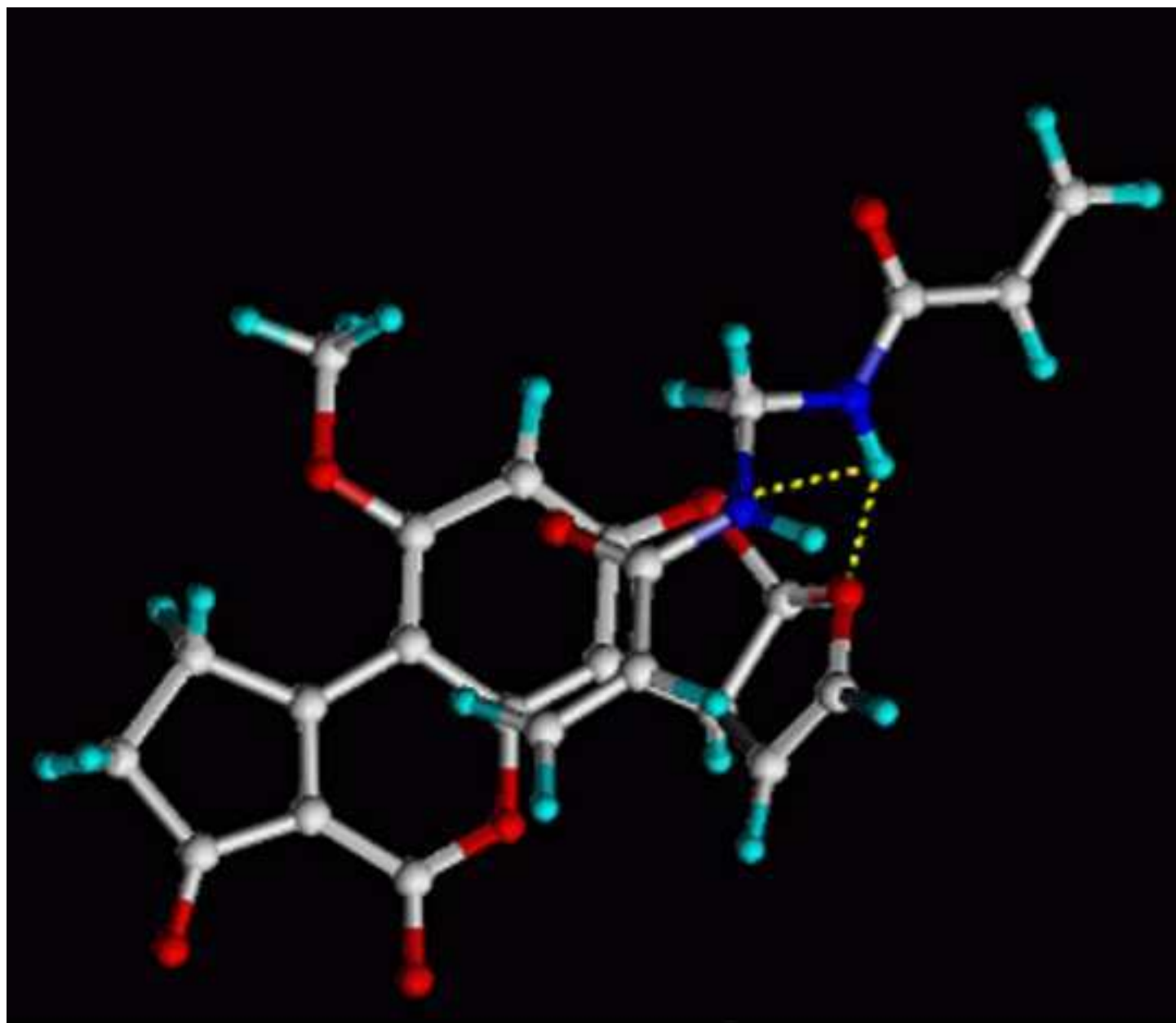
291 Figure 7. Calibration curve for quantification of OTA using the ToxiQuant System.

292 Figure 8. P2 polymer with 1 ng of adsorbed OTA (on the right), on the left- control
293 cartridge without toxin.



Figure 2





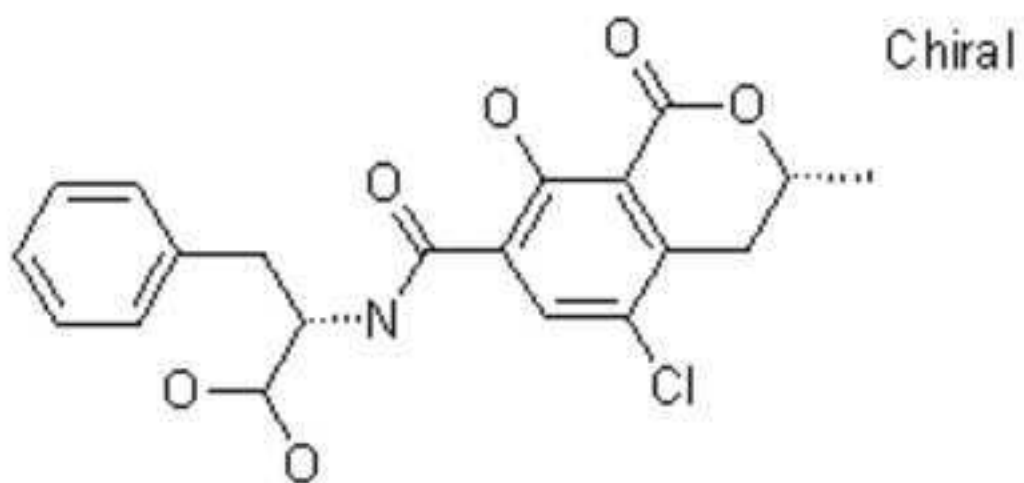
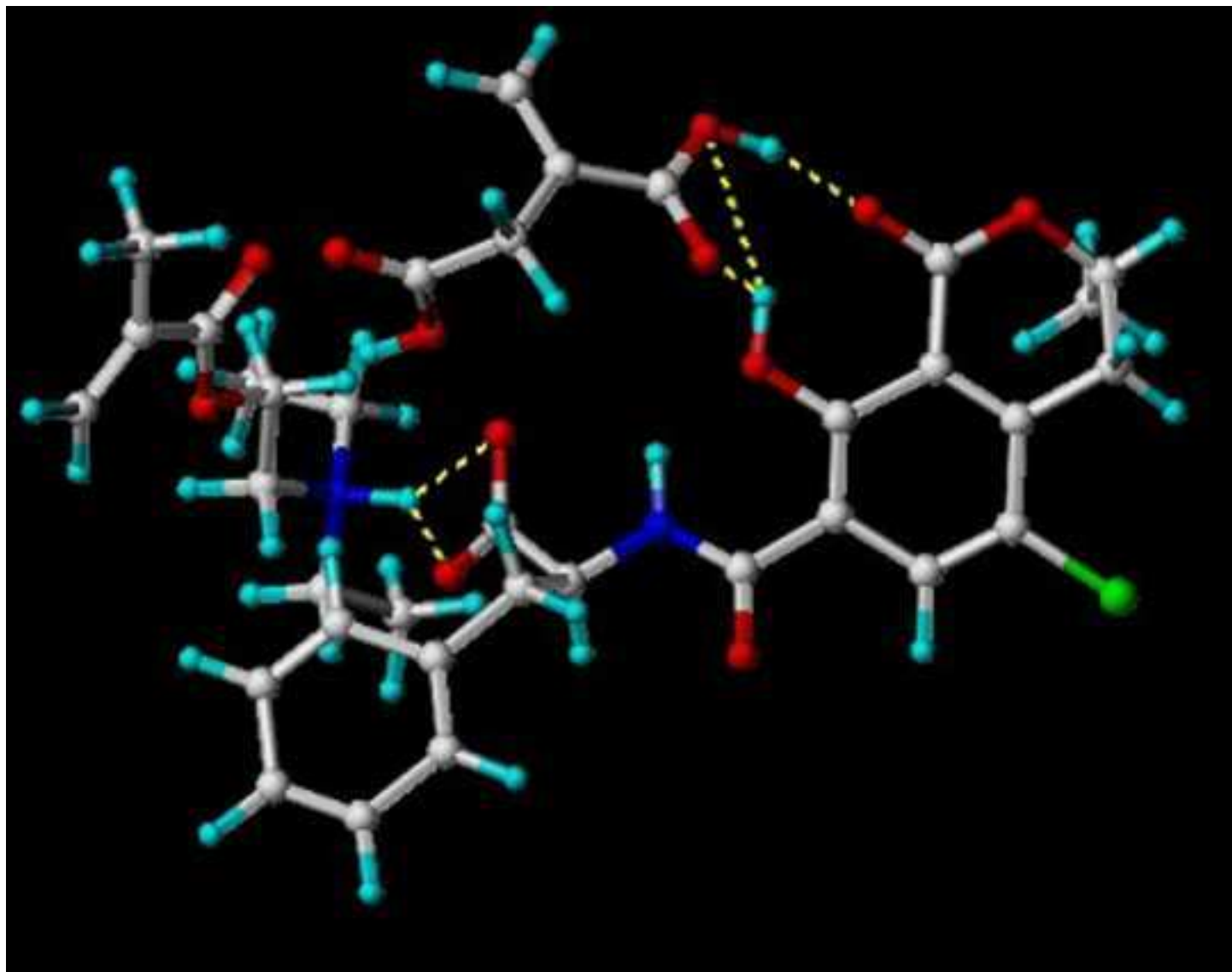
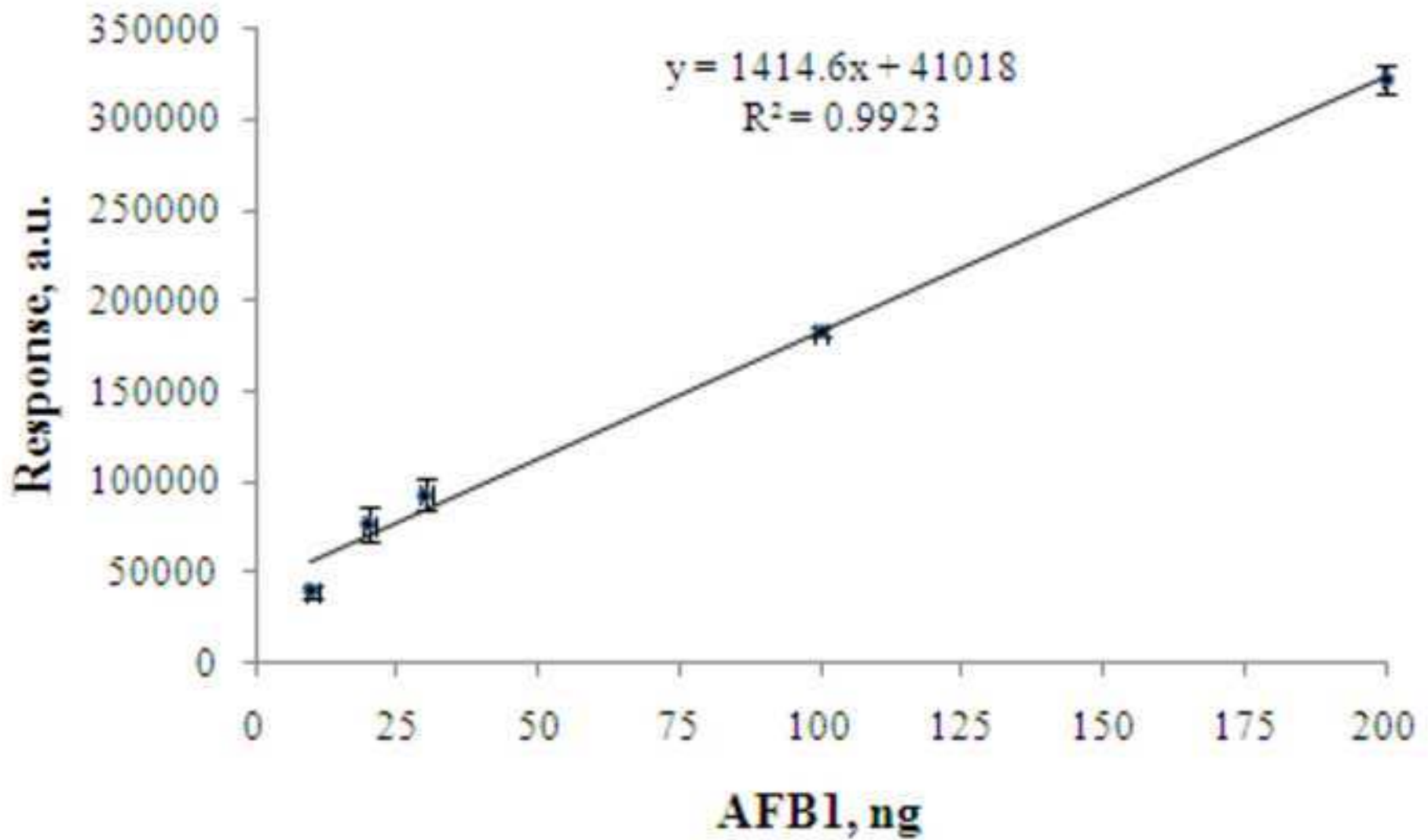


Figure 5



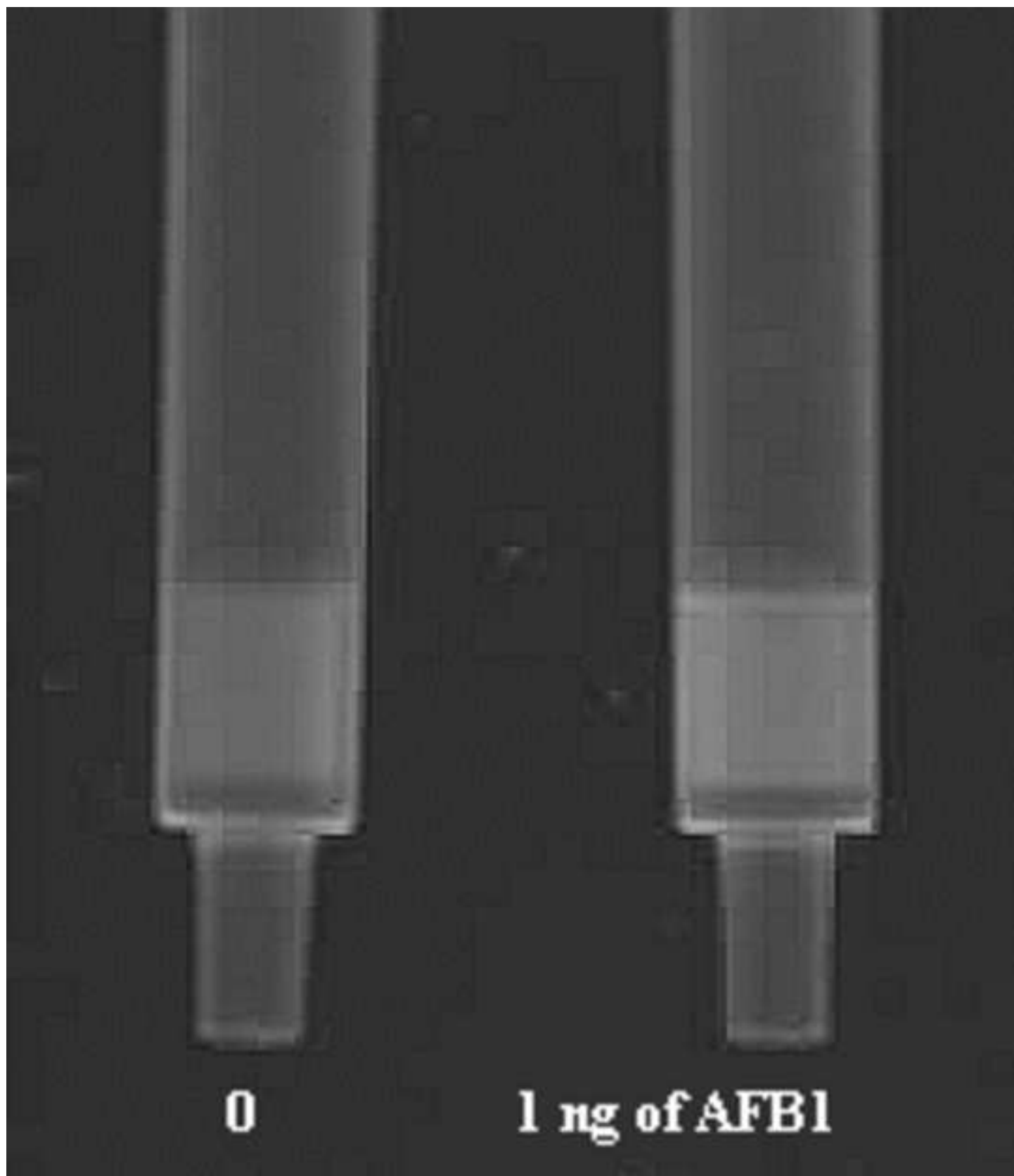


Figure 7

