1 **RAPID SCREENING ELECTROCHEMICAL METHODS FOR AFLATOXIN**
2 **B_1 AND TYPE-A TRICHOTHECENES: A PRELIMARY STUDY.**
3
4 *S. Piermarini*, *G. Volpe*, *F. Ricci, L. Micheli*, *D. Moscone, G. Palleschi**, [+]*M. Führer,*
5 [+]*R. Krska,* [+]*S. Baumgartner.*
6 Dipartimento di Scienze e Tecnologie Chimiche, Università degli Studi di Roma
7 “Tor Vergata”, Roma (Italy).
8 [+]Department for Agrobiotechnology (IFA-Tulln), University of Natural Resources
9 and Applied Life Sciences, Vienna (Austria).
10
11
12
13
14
15
16 [*]Corresponding author.
17 Tel.: +39 0672594423
18 Fax: +39 06 72594328
19 E-mail address: giuseppe.palleschi@uniroma2.it

20 **ABSTRACT**

21 In this work are presented methods for detection of Aflatoxin B_1 and type-A
22 trichothecenes, based on the use of indirect competitive ELISA format coupled with
23 a 96-well screen-printed microplate.
24 Electrochemical immunoassays for AFB_1, T-2 and HT-2 were performed and the
25 activity of the alkaline phosphatase or horseradish peroxidase labelled enzymes were
26 measured using Intermittent Pulse Amperometry as electrochemical technique.
27 Using standard solutions of the target analyte the LOD of the assays were 0.3 and 0.2
28 ng mL^{-1} for T-2 and AFB_1 respectively, while the sensitivity was 1.2 ng mL^{-1} for
29 both.
30 For Aflatoxin B_1, a stability study of electrochemical plate was also carried out.
31 Moreover, the matrix effect was evaluated using two different extraction treatments
32 from corn.
33

34 *Keywords: Aflatoxin B_1, Type-A trichothecene, 96-well screen-printed microplate,*
35 *competitive ELISA.*
36
37
38
39
40
41

42 **1. INTRODUCTION**

43 Mycotoxins are low-molecular-weight natural products produced as secondary
44 metabolites by filamentous fungi. Aflatoxins, trichothecenes, zearalenone,
45 fumonisins, and patulin, are the most important for their public health and
46 agroeconomic significance (Bennett and Klich 2003).

47 Aflatoxin B_1 (AFB_1), the most intensively studied among the mycotoxins (because of its
48 carcinogenic effects), is a metabolite mainly produced by the fungi *Aspergillus flavus*
49 and *A. parasiticus*. For this toxin, the European Commission set the maximum level for
50 AFB_1 in food to 2 ng g^{-1} (European Commission 1998).

51 Mainly produced by *Fusarium* fungi, which infect cereals in the field, Trichothecenes
52 have been associated with outbreaks of diseases both in humans and in farm animals.
53 They are often classified as Group A and Group B compounds depending on whether
54 they present a side chain on the C7 atom. Most important Trichothecenes are T-2 and
55 HT-2 for group A and DON and NIV for group B. For type A trichothecene the
56 maximum levels in food have not been still established, but the Joint FAO/WHO Expert
57 Committee on Food Additives (JECFA) has concluded that the toxic effects of T-2 toxin
58 and its metabolite HT-2 toxin could not be differentiated, and that the toxicity of T-2 'in
59 vivo' might be due at least partly to toxic effects of HT-2. Hence, HT-2 was included in
60 the provisional maximum tolerable daily intake (PMTDI), fixed at 60 ng Kg^{-1} body
61 weight per day of T-2 and HT-2 toxins, alone or in combination (Joint FAO/WHO
62 Expert Committee on Food Additives, 2001).

63 Contamination of cereals and related products with mycotoxins causes food- and feed-
64 borne intossication and carcinogenic effects in humans and farm animals (Krska et.
65 2001).
66 Commonly used methods for Trichothecenes and Aflatoxin detection include separating
67 techniques coupled to different detectors such as GC/FID, GC/ECD, GC/MS,
68 HPLC/UV, HPLC/FL, HPLC/MS (Schothorst and Jekel 2003; Milanez et al. 2006;
69 Klotzel et al. 2006; Jaimenez et al. 2000; Stroka and Anklam 2002). However, these
70 methods require specialized personnel and are very expensive. Several alternative
71 methods have been recently proposed for a rapid detection of these contaminants
72 including thin layer chromatography (Gilbert 1999; Snyder 1986), enzyme linked
73 immunosorbent assay (Piermarini et al. 2006; Ammida et al. 2004; Lee et al. 2004;
74 Kawamura et al. 1990; Ridascreen) and flow through immunoassays (Sibanda et al.
75 2000) (only for trichotecenes).
76 'Rapid tests' for mycotoxin analysis have been increasingly demanded by analytical
77 community, especially during the last decade.
78
79 This paper is focused on the development of Multichannel Electrochemical
80 Immunosensors (MEI), using a 96-well screen-printed microplate, for AFB_1, T-2 and
81 HT-2 detection. Preliminary studies are presented. A competitive indirect scheme for
82 detection of the target analytes was performed using monoclonal antibodies. At the
83 end of all immunological reactions, the enzymatic substrates (TMB or 1-NP), used

84 for signal generation, were added in each well and their conversion to electroactive
85 products was measured using Intermittent Pulse Amperometry (IPA) technique.
86

87 **2. EXPERIMENTAL**

88 **2.1 Reagents and materials**

89 Polystyrene microtitre plates, MaxiSorp, were purchased from NUNC (Roskilde,
90 Denmark). Mouse monoclonal antibody against aflatoxin B_1 (MAb) 5.4 mg mL^{-1} was
91 from HyTest Ltd (Finland). aflatoxin B_1, aflatoxin B_1-Bovine Serum Albumine
92 conjugate (AFB_1-BSA), polyvinylalcohol (PVA), polyoxyethylene sorbitan
93 monolaurate “Tween 20” (Tw20), 3,3’,5,5’-tetramethylbenzidine were from Sigma-
94 Aldrich Co. (St. Louis, MO, USA). T-2-Keyhole Limpet Hemocyanin (T-2-KLH, 0.5
95 mg mL^{-1}), mouse monoclonal antibody against T-2 toxin (clones: T-2C6 0.39 mg mL^{-1},
96 T-2B2 0.5 mg mL^{-1}, T-2B1 0.7 mg mL^{-1}), were kindly supplied by partners of the
97 BIOCOP project, while T-2, HT-2, DON and NIV were purchased from Biopure (Tulin,
98 Austria).
99 1-naphthylphosphate-disodium salt (1-NP), sodium chloride, potassium chloride,
100 magnesium chloride and diethanolamine (DEA) were provided by Fluka Chemie
101 (Sigma-Aldrich, Milan, Italy). Affinity-purified goat anti-mouse IgG (H+L) alkaline
102 phosphatase conjugate (Ab_2-AP) and nonfat dry milk blotting grade were from Bio-Rad
103 Laboratories (Hercules, CA, USA). Peroxidase anti-mouse IgG (H+L) affinity-purified
104 (Ab_2-HRP, 1 mg mL^{-1}) was purchased from Vector Laboratories Inc. (Burlingame, CA,
105 USA). Methanol and n-hexane were obtained from Riedel-dehaen (Sigma-Aldrich

106 Laborchemikalien). Corn samples were purchased from local supermarkets. Glass
107 microfiber filter (pore size 1.0 µm) from Whatman International (Ltd Maidston
108 England) and syringe filter glass fibre Acrodisc (1.0 µm, 37 mm), Pall Life Science,
109 from Sigma Aldrich.

110 **2.2 Apparatus**

111 A model 550-Microplate Reader (Bio-Rad Labs.) was used to read the absorbance on
112 ELISA plates at 405 nm.

113 The 96-well screen-printed microplates were obtained from Alderon Bioscience
114 Incorporated (Durham, USA). Working graphite electrodes (∅ 3 mm) with silver
115 reference electrode, screen-printed on a 0.5 mm plastic substrate, formed the two-
116 electrode system used (Fig. 1a). The plate is connected to the electrochemical reader
117 through a 56 dual positions card edge connector.

118 The electrochemical 96-well microplate reader (AndCare 9600) operates using
119 Intermittent Pulse Amperometry. IPA measurement on the AndCare 9600 sensor reader
120 (Fig. 1b) instrument involves a series of millisecond pulses of the same potential applied
121 individually to each of the 96 sensing electrodes.

122 By using Ab_2-AP and 1-NP (as enzymatic substrate), IPA measurements were carried
123 out at an applied potential of + 400 mV with a pulse width of 1 ms and a selected
124 frequency of 50 Hz. By using Ab_2-HRP and TMB (as enzymatic substrate), the applied
125 potential was –100 mV, pulse width and frequency were 10 ms and 5 Hz, respectively.
126 The results were recorded on a PC using dedicated software.

127 Further laboratory equipment includes an Autovortex SA6 (Stuart Scientific, UK),
128 high-speed blender Osterizer (Sunbeam), a horizontal shaker (Instruments s.r.l-Milan-
129 Italy) and a centrifuge Mod PK 120 (ALC(R)-Tecnochimica Moderna s.r.l.-Italy).
130
131 **2.3 Procedures**
132
133 *2.3.1 T-2, HT-2: procedure for spectrophotometric ELISA*
134 The microtitre plate wells were coated overnight by incubation at 4 °C with 200 μL of
135 conjugate T-2-KLH solution (2 μg mL^{-1} for T-2B1 clone, 5 μg mL^{-1} for T-2B2 and T-
136 2C6 clones) in pH 9.6 carbonate buffer solution, and then blocked (1 h at RT=room
137 temperature) with a solution of 3% dry milk.
138 After blocking, a one step competition was performed as following: 100 μL of standard
139 solutions were added in triplicate to the wells, followed by 100 μL of MAb anti-T-2
140 diluted 1:1000 v/v, 1:1000 v/v, 1:100 v/v for T-2B1, T-2B2, T-2C6 clones, respectively.
141 The competition was performed for 2 h at RT. The antibody-toxin-KLH complex was
142 revealed using affinity purified peroxidase-labelled goat anti-mouse IgG (200 μL/well)
143 diluted 1:1000 v/v (1 h at RT). Between each step (coating, blocking and competition) a
144 three-cycle washing procedure using 0.05% Tween 20 in PBS (PBS-T) was adopted.
145 Each solution, except the coating and blocking ones, was prepared in PBS. Finally, a
146 stock solution of TMB (20 mM) in DMSO was prepared and 200 μL of substrate
147 solution (3×10^{-4} M TMB + 10^{-3} M H_2O_2 in 0.05 M citrate phosphate buffer + 0.1 M

148 KCl, pH = 5.0) were added to each well; the enzymatic reaction was stopped after 5 min
149 by adding 50 μL of 2 M H_2SO_4, and the absorbance was read at 405 nm.
150
151 *2.3.2 T-2: MEI procedure*
152 The screen-printed wells were pre-wetted with 200 μL of deionised water prior to the
153 coating deposition. The surfaces of the screen-printed electrodes were then coated with
154 80 μL of T-2-KLH (2 μg mL^{-1} in carbonate buffer, pH 9.6) and incubated overnight at
155 4 ^{o}C. The well surfaces were then blocked by adding 80 μL of 1% dry milk (prepared in
156 carbonate buffer) and incubated for 15 min at RT.
157 A one step competition was performed, using only the clone T-2B2 (selected in the
158 spectrophotometric assay), as following:
159 40 μL of anti-T-2 MAb (1:1250 v/v in PBS) and T-2 standard solutions (prepared in
160 PBS) were added into each well and the competition, between free and coated analyte,
161 was performed for 30 min at RT. The antibody-toxin-KLH complex was revealed using
162 Ab_2-HRP (80 μL/well) diluted 1:500 v/v (30 min at RT). Between the coating, blocking,
163 competition and labelling, three washing steps were carried out, twice with PBS-T and
164 once with only PBS.
165 Each solution, except the coating and blocking ones, was prepared in PBS. Finally, 80
166 μL of a substrate solution, prepared as reported in the spectrophotometric procedure,
167 was added to each well and the current was recorded after 2 min at RT.
168 The amount of the enzymatic product (TMB_{red}) formed was detected by IPA, at an
169 applied potential of –100 mV.

170 *2.3.3 Aflatoxin B_1: MEI procedure*

171 Also in this case, the screen-printed wells were pre-wetted. The surfaces of the screen-
172 printed electrodes were then coated with 80 µL of AFB_1-BSA (1µg mL^{-1} in carbonate
173 buffer, pH 9.6) and incubated overnight at 4°C. The well surfaces were then blocked by
174 adding 80 µL of 1% PVA (prepared in carbonate buffer) and incubated for 30 min at
175 RT.

176 A two-step competition was performed as following:

177 In the first step, equal volumes of anti-AFB_1 MAb diluted 1:13500 v/v in PBS (which
178 corresponds to a concentration of 0.4 µg mL^{-1}) and standard AFB_1 solutions were mixed
179 and allowed to react at RT for 10 min. In the second step, 80 µL of this mixture were
180 added into each well of the microplate and the competition, between free and coated
181 analyte, was performed for 15 min at RT. The labelling step was carried out by adding
182 80 µL of Ab_2-AP (1:100 v/v in PBS) and incubating for 5 min at RT. After each step,
183 three washings were carried out such as in T-2 MEI procedure. Finally, 80 µL of 1-NP
184 substrate (5 mg mL^{-1} in 0.97 M DEA buffer + 1 mM $MgCl_2$ + 0.15 M KCl, pH 9.8)
185 were added into each well and allowed to react for 1 min at RT. The amount of
186 enzymatic product (1-naphthol) formed was detected by IPA, at an applied potential of
187 + 400 mV.

188

189 *2.4 Calibration graphs*

190 The standard curves were fitted using "non-linear 4 parameter logistic calibration
191 plots" (Warwick, 1996). The four parameter logistic function is:

192 $$f(x) = \frac{a-d}{1+(x/c)^b} + d$$

193 where *a* and *d* are the asymptotic maximum and minimum values, respectively; *c* is the
194 value at the inflection point and *b* is the slope.

195 The detection limit (LOD) was defined as the concentration corresponding to the f(x)
196 value obtained by subtracting three standard deviations of zero point (no analyte) from
197 the mean of the zero point measurements (mean value-3sd) (Law and Biddlecombe,
198 1996).

199 Cross-reactivity of the antibodies (anti-AFB_1 and anti-T-2) against other mycotoxins
200 was used to determine the specificity of the assays. Cross-reactivity was defined as 100
201 *x*/*y*, were *x* is the amount of the target analyte and *y* is the amount of interfering
202 mycotoxins required to produce 50% inhibition of the binding between MAb and
203 coated-toxin (AFB_1-BSA, T-2-KLH).

204

205 *2.5 Corn sample treatment*

206 Two different corn sample treatments were carried out and only the matrix effect on
207 the performance of the MEI system was evaluated.

208 Non-infected corn kernels were first ground in a household blender at high speed for 1
209 min.

210 The first procedure employed for sample treatment (S. Piermarini et al. 2006) is the
211 following:

212 • weight 10 g of corn powder;

213 • add 50 mL of extraction solvent (85% methanol in PBS) and mix for 45 min in
214 a horizontal shaker;
215 • centrifuge at 6000 rpm for 10 min;
216 • dilute 2 mL of the supernatant with 8 mL of PBS;
217 • defatted for 5 min with 5 mL *n*-hexane;
218 • after separation of the two layers, the aqueous layer was recovered.
219
220 The second procedure, employed for sample treatment, was a modification of that
221 reported by the kit "I'screen $AflaB_1$ gold" (Tecna, Trieste, Italy):
222 • weight 50 g of corn powder;
223 • add 5 g of NaCl;
224 • add 100 mL of a solution of methanol 80% in distilled water and mix
225 thoroughly for 3 min a high-speed blender;
226 • filter (glass microfiber filter pore size 1.0 μm);
227 • mix 2 mL of filtrate with 8 mL of PBS;
228 • filter (syringe filter glass fibre Acrodisc, 1.0 μm).
229 In order to evaluate the matrix effect, at the end of both procedures, standard solutions
230 of AFB_1 were added to sample extracts and analysed by the MEI system.
231
232
233
234

235 **3. RESULTS AND DISCUSSION**

236

237 *3.1 Type-A Trichothecenes detection.*

238 *3.1.1 Spectrophotometric assay*

239 Because the JECFA has fixed a provisional maximum tolerable daily intake of 60
240 ng Kg^{-1} body weight per day for T-2 and HT-2 toxins, alone or in combination, it is
241 important to develop a system for the simultaneous detection of both toxins.
242 For this reason, a spectrophotometric test using different monoclonal antibodies
243 anti-T-2 toxin (produced by three different clones) has been accomplished in order to
244 select those with the greatest cross-reactivity for HT-2 toxin.
245 For each clone, binding curves using different concentrations (1, 2 and 5 μg mL^{-1})
246 of the coated molecule (T-2-KLH) and serial dilutions of MAb (1:100 – 1:50000 v/v)
247 were constructed in order to fix the best conditions for the competitive ELISA. These
248 experiments were performed as reported in the section 2.3.1 but, in this case, only 200
249 μL of MAb in PBS (instead of 100 μL of MAb and 100 μL of free analyte) were
250 added in triplicate to the wells. From the binding curves (data not shown) it was
251 established that 2 μg mL^{-1} (for T-2B1 clone) and 5 μg mL^{-1} (for T-2B2 and T-2C6
252 clones) of T-2-KLH gave the best absorbance signals; while for MAb anti-T-2
253 dilutions of 1:2000 v/v, 1:2000 v/v, 1:200 v/v (for T-2B1, T-2B2, T-2C6 clones,
254 respectively), corresponding to the inflection points of the three binding curves, were
255 selected.

256 Typical sigmoid calibration curves for T-2 toxin, obtained with the three clones, are
257 reported in Fig. 2.
258 The detection limit was calculated to be 0.7, 0.6 and 0.3 ng mL^{-1} for T-2B1, T-2B2
259 and T-2C6 respectively. Sensitivities, calculated as the amount of T-2 toxin to
260 produce a 50% decrease in the signal were 1.8, 2.3 and 1.0 ng mL^{-1} for T-2B1, T-2B2
261 and T-2C6, respectively. The analysis time was about 4.5 h.
262 The cross-reactivity of the three MAb clones for HT-2 and the most common type-
263 B trichothecenes (DON and NIV) was also evaluated, carrying out the calibration
264 curves for all toxins. For each cross-reactant the corresponding amount required to
265 produce 50% inhibition of the binding was calculated, and the relative cross-reactivity
266 of the tested toxins is presented in Table 1. As shown the T-2B2 clone gave the
267 highest cross-reactivity (62%) towards HT-2 toxin while no appreciable interaction
268 with type-B trichothecenes was observed. For this reason, T-2B2 clone was selected
269 to develop the MEI system.
270
271 *3.1.2 MEI assay*
272 Also in this case binding curves were constructed using different dilutions of the
273 selected MAb (T-2B2 clone) and two different concentrations of the coated molecule
274 (T-2-KLH) in order to establish the best conditions for the competitive indirect
275 ELISA.
276 These experiments were performed as reported in section 2.3.2 but, in this case, 80
277 µL of MAb in PBS (instead of 40 µL of MAb and 40 µL of free analyte) were added

278 in triplicate to the wells. Also, to evaluate the non-specific adsorption of the
279 monoclonal antibody to the electrode surface, the same MAb dilutions were added to
280 the blocked screen-printed-wells but without T-2-KLH. As shown in Figure 3, the
281 best conditions were obtained using a dilution of 1:2500 v/v for MAb and a
282 concentration of 2 μg mL^{-1} for T-2-KLH. Non-specific adsorptions was not observed.
283 The dilutions chosen above, for MAb and conjugate, were used for further
284 competitive assay. The standard curve generated using these optimized conditions is
285 shown in Figure 4. The detection limit and the sensitivity were 0.3 and 1.2 ng mL^{-1}
286 respectively, while the analysis time was about 1.5 h.
287 Although this system is very rapid and easy to use for the analysis of T-2 and HT-2
288 trichothecene, it is still under study because we are screening new monoclonal
289 antibodies and we have obtained promising results with a MAb clone produced against
290 HT-2 toxin, that appears to give a 97% of cross-reactivity with T-2 toxin. Only in this
291 way it will be possible to obtain an accurate quantification of the total amount of T-2
292 and HT-2 toxins.
293
294 *3.2 Aflatoxin B_1: MEI assay*
295 This part of the work represents a continuation and improvement of our previous
296 studies using a multichannel electrochemical 96-wells plate coupled with polyclonal
297 antibody for aflatoxin B_1 determination in corn (Piermarini et al. 2006). In these
298 previous studies the cross-reactivity of the PAb towards different aflatoxins has been
299 evaluated, showing a value of 56% for AFG_1, while in another paper (Ammida et al.

300 2006) the same antibody gave a value of 112% for AFG_1. These experiments showed
301 that there was considerable variation between different lots of polyclonal antibody, so
302 all further experiments, presented in this paper, were carried out using monoclonal
303 antibodies, produced from a single clone against AFB_1. Also in this case, the cross-
304 reactivity of the MAb, toward other aflatoxins, was evaluated showing a high specificity
305 for AFB_1.
306 Such as for trichothecene assay, binding curves using different concentrations of AFB_1-
307 BSA and serial dilutions of MAb were performed. Figure 5 reports the chosen titration
308 curve obtained with 1 μg mL^{-1} of the coated molecule. We select a dilution of 1:27000
309 v/v (corresponding to a concentration of 0.2 μg mL^{-1}), which represents the inflection
310 point of the curve. By using the procedure reported in section 2.3.3, a calibration curve
311 for AFB_1 (labelled with closed circles in Figure 7) was carried out. LOD and sensitivity
312 were calculated to be 0.2 and 1.2 ng mL^{-1} respectively, with an analysis time of about 1
313 h.
314 The stability of the coated and blocked plate (dry stored at 4° C) was evaluated by
315 comparing calibration curves performed periodically during one month. As shown in
316 Figure 6, the immunoplate can be used until 30 days of its preparation without lost of
317 sensitivity. Moreover, considering the possibility to prepare and store the coated and
318 blocked plates, the analysis time can be reduced at 30 min .
319 For future applications of this system to real samples, the matrix effect was evaluated
320 using two different extraction treatments from corn, as reported in section 2.5.

321 In Figure 7 are reported calibration curve in PBS compared with those obtained by
322 adding the same concentrations of AFB_1 (0-100 ng mL^{-1}) to 1:5 v/v diluted blank corn
323 extracts. The results indicated a negligible matrix effect, with the advantages that the
324 second procedure is more simple and rapid than the first one.
325

326 **4.CONCLUSIONS**

327 In this paper preliminary studies about the development of two immuno-
328 electrochemical systems, based on the use of a 96-well screen-printed plate, for
329 detection of AFB_1, T-2 and HT-2 toxins are presented.

330 Both tests were found to be simple, rapid, specific for the target analyte, and could
331 allow simultaneous analysis of numerous samples. Only after a complete
332 characterization and application of these systems to a large number of cereal samples,
333 experimentally and naturally contaminated, they could be considered a future trend in
334 the screening analysis of mycotoxins.
335

336 **Acknowledgements**

337 The authors wish to thank the Italian projects MIPAF ‘Aflarid’, PRIN-2005, the
338 European project ‘Biocop’, and the EUROLAB S.r.l. for financial support.
339

340 **REFERENCES**

341 Ammida, N.H.S., Micheli, L., and Palleschi, G. 2004. Electrochemical
342 immunosensor for determination of aflatoxin B1 in barley. *Anal. Chim. Acta,* 520 :
343 159-164.
344 Ammida N.H.S., Micheli L., Piermarini S., Moscone D., Palleschi G. 2006.
345 Detection of aflatoxin B_1 in barley: comparative study of immunosensor and HPLC.
346 *Anal. Letters*, 39 : 1559-1572.
347 Bennett, J.W. and Klich, M. 2003. Mycotoxins. *Clin. Microbiol. Rev.,* 16 (3): 497-
348 516.
349 European Commission, Commission Regulation (98/53/EC) 1998. Official Journal of
350 European Communities 1998 L201/93.
351 Gilbert, J. 1999. Overview of mycotoxin methods, present status and future needs.
352 *Nat. Toxins,* 7: 347-352.
353 Jaimenez, J., Fent, C., Vazquez, B., Franco, C., Capeda, A., Mahuzier, G., and
354 Prognon, P. 2000. Application of the assay of aflatoxins by liquid chromatography
355 with fluorescence detection in food analysis-a review. *J. Chromatogr. A*, 882: 1-10.
356 Kawamura, O., Satoshi, N., Sonomi, S., Katsumi, O., Yoshitugu, S., Toshitugu, T.,
357 Yoshio, U. 1990. Survey of T-2 toxin in cereals by an indirect enzyme-linked
358 immunosorbentassay. *Food Agric. Immunol.*, 2: 173-180.
359 Klotzel, M., Lauber, U., and Humpf, H.U. 2006. A new solid phase extraction clean-
360 up method for the determination of 12 type A and B trichothecenes in cereals and
361 cereal-based food by LC-MS/MS. *Mol. Nutr. Food Res.* 50: 261-269.

362 Koch, P. 2004. State of the art of trichothecenes analysis. *Toxicol. Lett.,* 153: 109-
363 112.
364 Krska, R., Baumgartner, S., Joseph, R. 2001. The state of the art in the analysis of type-
365 A and –B trichothecene mycotoxins in cereals. *Fresenius J. Anal. Chem.*, 371: 285-
366 299.
367 Law, B. and Biddlecombe, R.1996. Validation of an immunoassay. In *Immunoassay, a*
368 *practical guide*; Law, B. (ed.); Taylor & Francis Ltd: UK.
369 Lee, N.A., Wang, S., Allan, R.D. and Kennedy, I.R. 2004. A rapid aflatoxin B1
370 ELISA: development and validation with reduced matrix effect for peanuts, corn
371 pistachio and soybeans. *J. Agric. Food Chem.,* 52: 2276-2755.
372 Milanez, T.V. and Valente Soares, M.L. 2006. Gas chromatography-mass
373 spectrometry determination of thrichotecene mycotoxins in commercial corn
374 harvested in the state of São Paulo, Brazil. *J. Braz. Chem. Soc.,* 17 (2): 412-419.
375 Piermarini, S., Micheli, Ammida, N.H.S, Palleschi, G., and Moscone, D. 2006.
376 Electrochemical immunosensor array using a 96-well screen-printed microplate for
377 aflatoxin B_1 detection. *Biosens. Bioelectron.* (in press).
378 Ridascreen T-2 toxin. Art Nr R3801 Enzyme immunoassay zur quantitativen
379 Bestimmung von T-2 Toxin, r-Biopharm GmbH, Darmstatd, Deutschland.
380 Schothorst, R.C. and Jekel, A.A. 2003. Determination of trichothecenes in beer by
381 capillary gas chromatography with flame ionisation detection. *Food Chem.,* 82:
382 475-479.

383 Sibanda, L., De Saeger, S., Van Peteghem, C., Grabarkiewicz-Szczesna, J., and
384 Tomczak, M. 2000. Detection of T-2 toxin in different cereals by flow-through
385 enzyme immunoassay with a simultaneous internal reference. *J. Agric. Food Chem.,*
386 48: 5864-5867.
387 Snyder, A. P. 1986. Qualitative, quantitative and technological aspects of the
388 trichothecene mycotoxins. *J. Food Protect.*, 49 (7): 544-569.
389 Stroka, J. and Anklam, E. 2002. New strategies for the screening and determination
390 of aflatoxins and the detection of aflatoxin producing moulds in food and feed.
391 *TRAC-Trend. Anal. Chem.,* 21: 90-95.
392 Warwick, M. 1996. Standardisation of immunoassays. In *Immunoassay, a practical*
393 *guide*; Brian, L. (ed.); Taylor & Francis Ltd: UK, 150-170.
394 WHO Food Additives Series 47, FAO Food and Nutrition Paper 74, presented at the
395 56th Meeting of the Joint FAO/WHO Expert Committee on Food Additives
396 (JECFA), WHO Geneva, 2001, p. 557).
397
398
399
400
401
402
403
404

405
406 **Legend of figures**

407

408 **Fig. 1**: a) 96-well screen-printed microplate; b) AndCare 9600 sensor reader.

409 **Fig. 2:** spectrophotometric competition curves for T-2 toxin using different MAb
410 clones: T-2C6 (▼), T-2B2 (○) and T-2B1 (♦).

411 **Fig. 3:** binding curves obtained by MEI system using different dilutions of MAb (T-
412 2B2) and two concentrations of coated molecule (T-2-KLH): 2 μg mL^{-1} (●) and 0.5
413 μg mL^{-1} (▲). Blanks (○) carried out in the same experimental conditions but without
414 immobilization of T-2KLH.

415 **Fig. 4:** electrochemical standard curve for T-2 toxin.

416 **Fig. 5:** selected binding curve obtained by MEI using different dilutions of MAb and a
417 concentration of the coated molecule AFB_1-BSA = 1 μg mL^{-1}. No coating (○) carried
418 out in the same experimental conditions, using only MAb diluted 1:5400 v/v.

419 **Fig. 6:** stability studies of the coated and blocked plate performed periodically,
420 during 30 days.

421 **Fig. 7:** Effect of corn extracts on the standard curve of AFB_1 detected by MEI.
422 Standard solution of AFB_1 prepared in PBS (●), in non-infected corn extracted by the
423 first procedure (◊) and by the second procedure (Δ), both diluted 1:5 v/v with PBS
424 before the analysis.

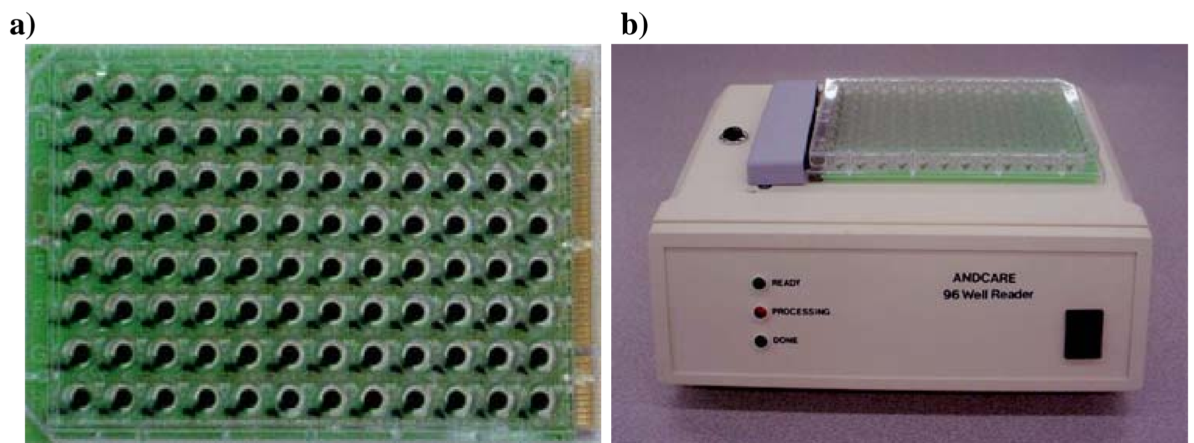
425
426
427

428
429
430
431
432
433
434
435
436 **Table 1.** Cross-reactivity % of three clones of monoclonal antibodies to different
437 trichothecenes.
438

Trichothecene	**Cross-reactivity (%)**		
	Clone T-2B2	Clone T-2B1	Clone T-2C2
DON	0	0	0
NIV	0	0	0
HT-2	62	37	33
T-2	100	100	100

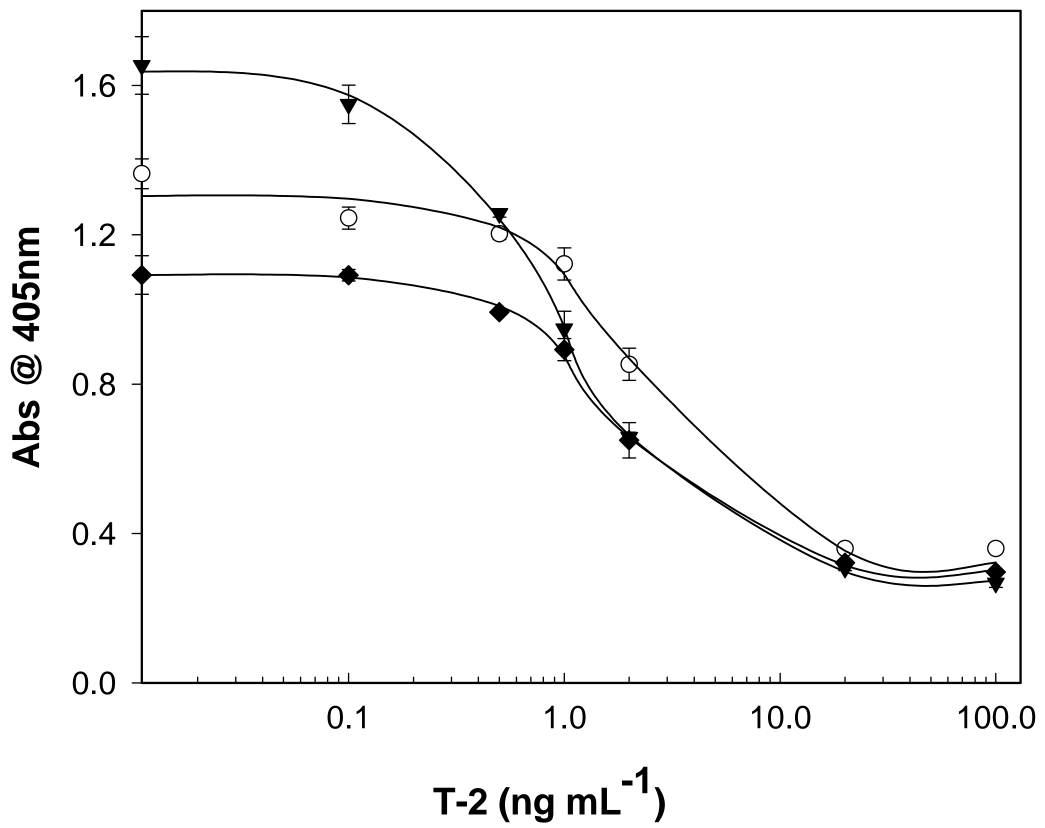
439
440
441
442
443
444
445
446
447
448
449
450
451
452
453
454
455
456
457
458

459
460
461
462
463
464
465
466
467
468
469
470
471
472
473
474 **a)** **b)**



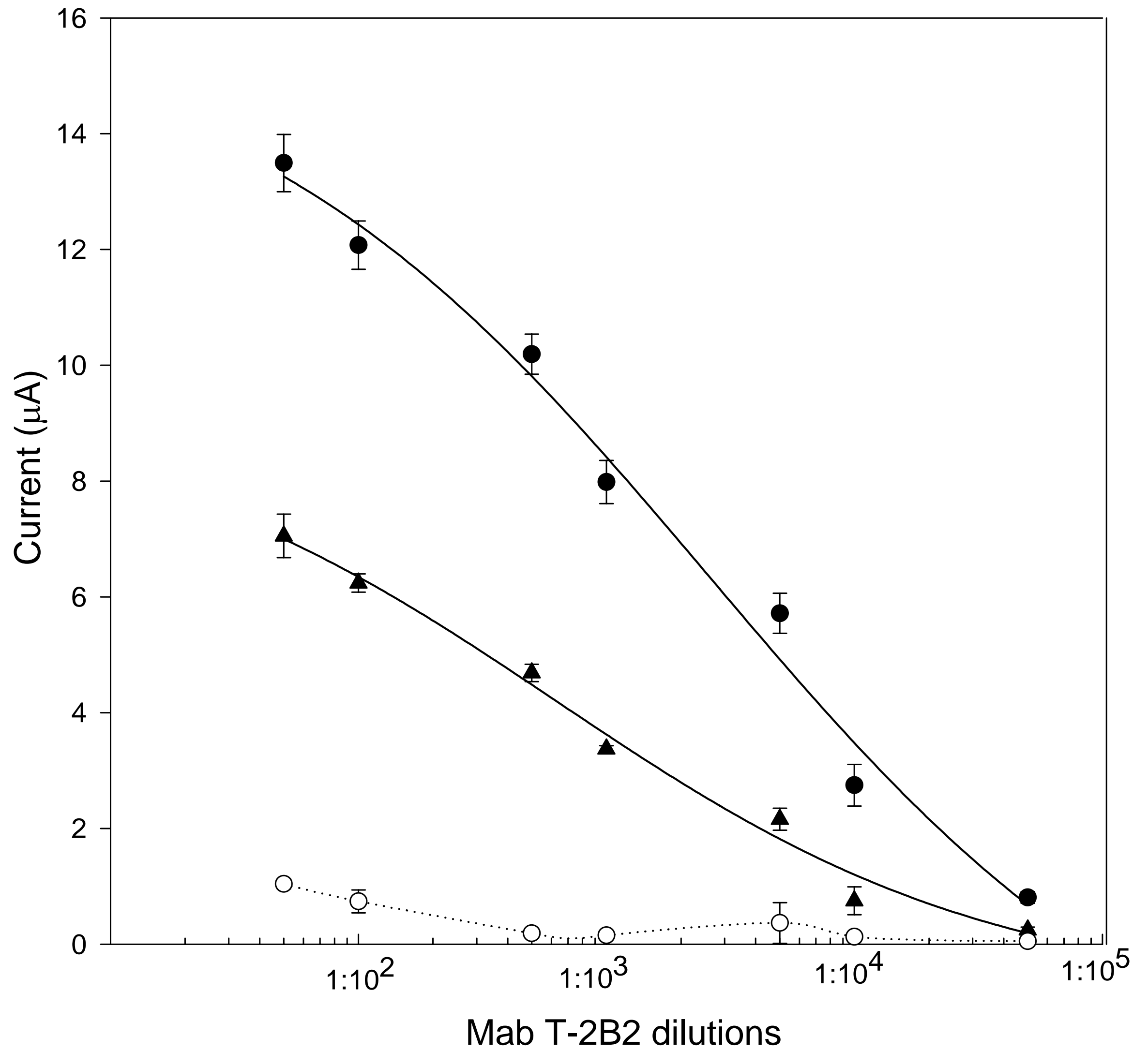
475
476
477
478
479
480
481
482
483
484
485
486
487
488
489
490
491 **Figure 1.**
492
493
494
495
496
497
498
499
500
501
502

503
504
505
506
507
508
509
510
511
512
513
514



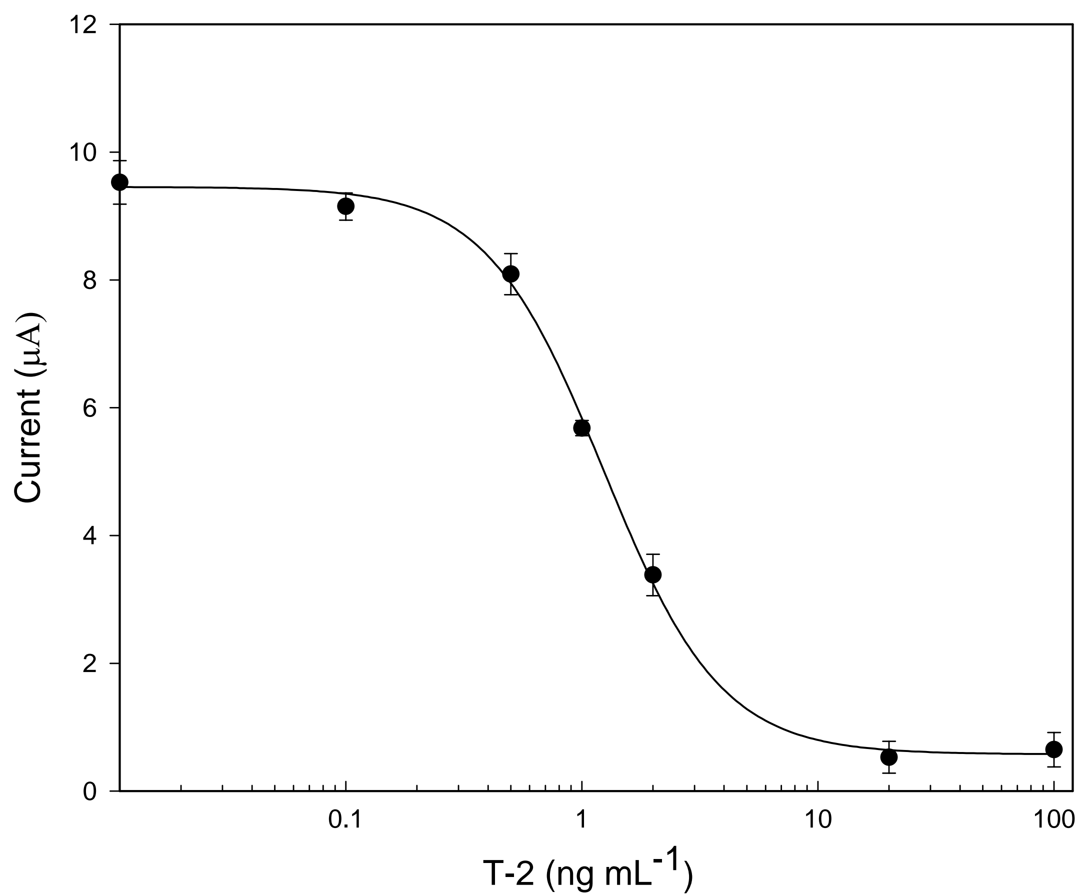
515
516
517
518
519
520
521
522 **Figure 2.**

523
524
525
526



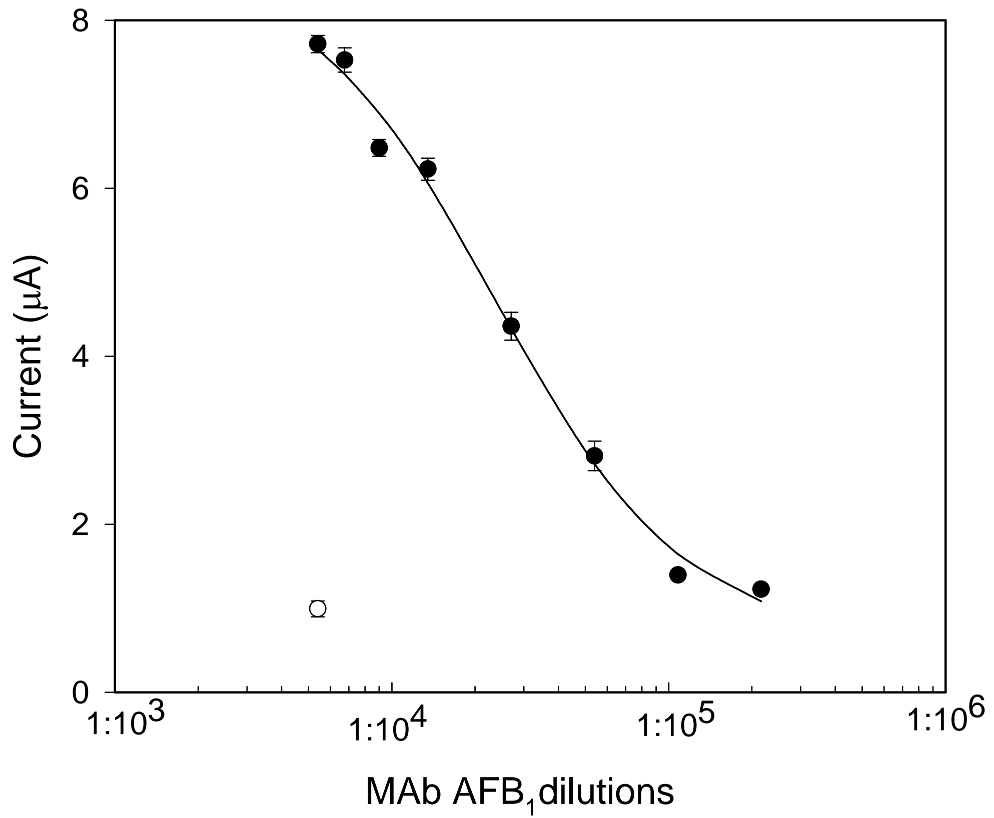
527
528
529
530
531
532
533 **Figure 3.**
534
535
536
537

538
539
540
541
542
543
544
545



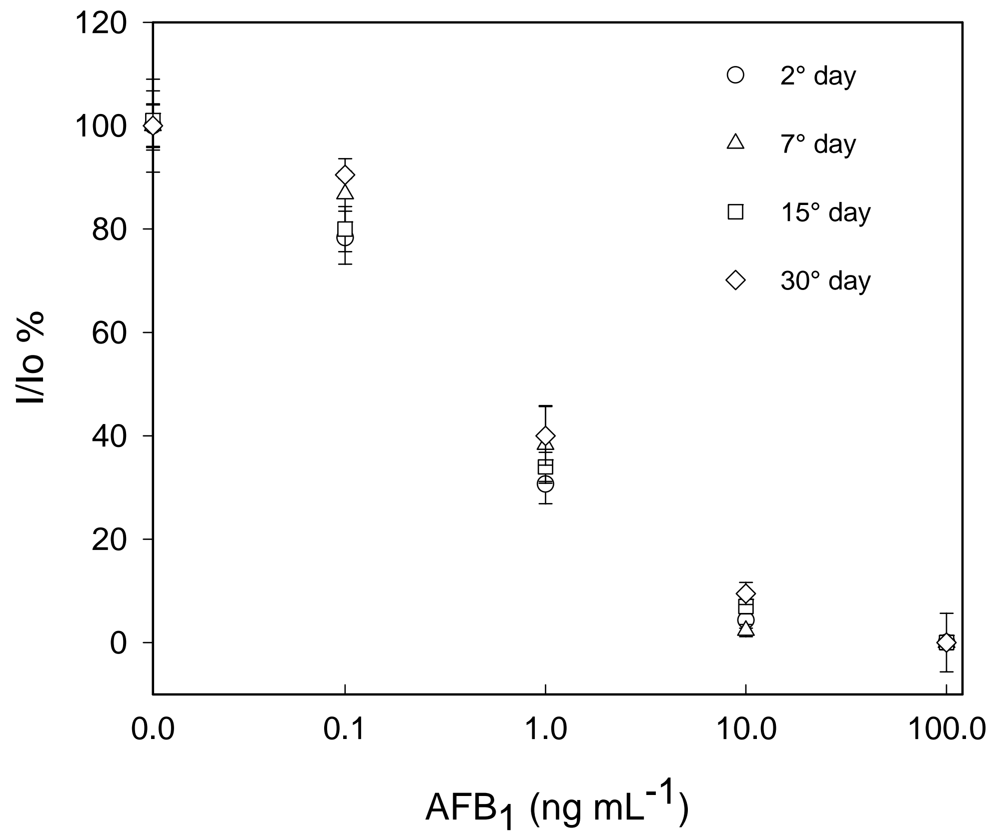
546
547
548
549
550
551 **Figure 4.**
552
553
554
555
556

557
558
559
560



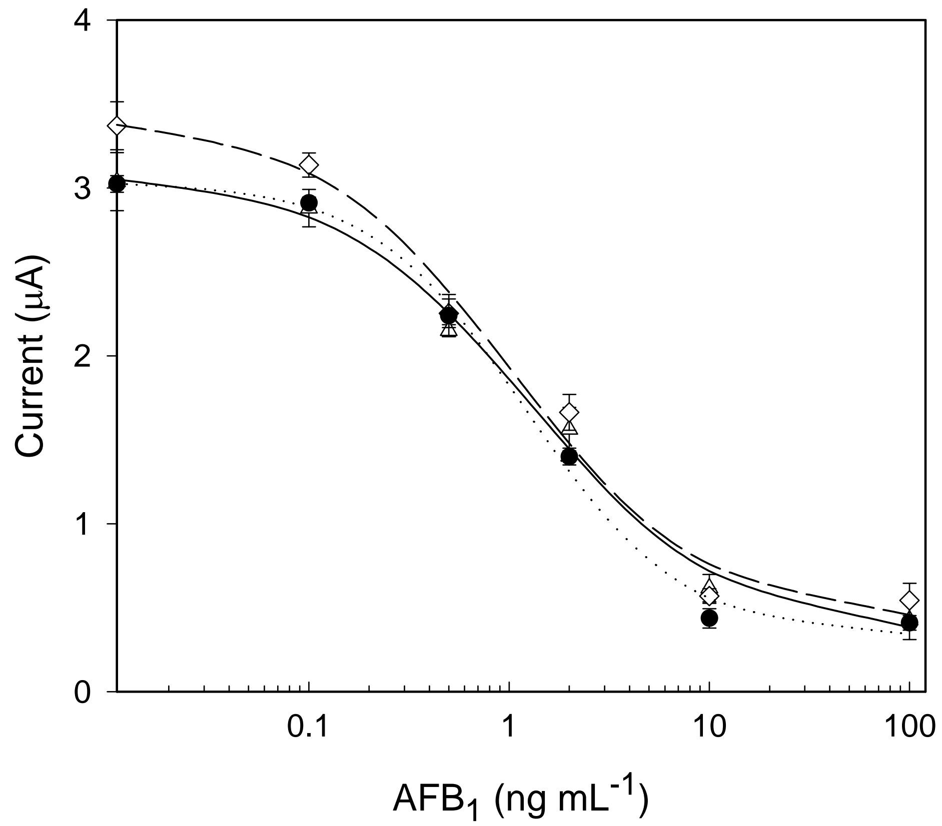
561
562
563
564
565
566
567 **Figura 5.**
568
569
570
571
572
573
574
575
576

577
578
579
580
581
582
583



584
585
586
587
588
589 **Figure 6.**
590
591
592
593
594
595
596

597
598
599
600
601
602



603
604
605
606 **Figure 7.**
607