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## Enzyme immunoassay for monitoring aflatoxins in eggs

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## UNIVERSITÀ DEGLI STUDI DI TORINO

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20 ENZYME IMMUNOASSAY FOR MONITORING AFLATOXINS IN EGGS

21

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29

### 30 ABSTRACT

31 Rapid and sensitive competitive enzymatic immunoassays for measuring most relevant aflatoxins in eggs  
32 have been developed by synthesizing two hapten derivatives. Polyclonal antibodies raised against a hapten  
33 obtained from aflatoxin B1 (AFB1) were exploited to set an AFB1-selective assay, whereas antibodies  
34 obtained through immunising with a hapten derived from aflatoxin M1 (AFM1) allowed us to detect four  
35 principal aflatoxins (B1, G1, B2, and G2) and the most relevant AFB1 metabolite (AFM1) with detection  
36 limits in eggs of 0.3  $\mu\text{g kg}^{-1}$  for AFB1, AFG1, and AFM1 and 3  $\mu\text{g kg}^{-1}$  for AFB2 and AFG2, respectively. We  
37 also established a rapid and simple protocol for extracting aflatoxins from eggs by employing aqueous  
38 methanol (70%) followed by partitioning with hexane to remove fats. The whole analytical process is  
39 simple, very rapid (the extraction requires 14 minutes, and the assay is completed in 30 minutes) and  
40 proved to be accurate and precise enough (recoveries ranged from 84 to 100% and RSD% were within 20%  
41 for intra- and inter-assay experiments) to be proposed as a first level screening method for the monitoring  
42 of the occurrence of aflatoxins in egg.

43

44 **KEYWORDS:** Aflatoxin M1, group-selective immunoassay, aflatoxin extraction

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52 **INTRODUCTION**

53 Aflatoxins are secondary metabolites produced by moulds of the *Aspergillus* family, which contaminate  
54 several crops, including cereals, oilseeds, tree nuts, and spices. Due to the fact that *Aspergillus* moulds  
55 could grow on crops pre-, during, and post-harvest and that their toxic metabolites are very stable to  
56 chemical and physical stresses, aflatoxins have been found in raw and processed materials and represent  
57 the most common cause of chemical contamination of foodstuffs, according to the European Union alert  
58 system (EU Rapid Alert System for Food and Feed). Among about 300 different natural aflatoxins, the most  
59 diffuse and toxic is the aflatoxin B1 (AFB1). It is produced by *A.flavus* and *A. parasiticus* and has been  
60 recognized as the most potent carcinogen for human (International Agency for Research on Cancer, 2002).

61 Besides AFB1, principal aflatoxins are: aflatoxin B2 (AFB2), which is produced by the same mould as AFB1  
62 but in a lesser extent; aflatoxin G1 (AFG1) and aflatoxin G2 (AFG2), which belonged to *A. parasiticus* (AFG1  
63 is the predominant toxin excreted by *A. parasiticus*). Maximum acceptable levels for AFB1 and for the sum  
64 of all the four aflatoxins have been set worldwide in commodities susceptible to contamination and  
65 intended for human consumption (European Commission, 2010) or for feeding farm animals (European  
66 Commission, 2003). Furthermore, it has been demonstrated that dairy cattle, sheep and goats fed with  
67 AFB1 contaminated feedstuffs transfer the aflatoxin to milk partially as the unmodified precursor, but  
68 primarily as a hydroxylated metabolic product (Van Egmond, 1989). This AFB1 metabolite excreted to milk  
69 (aflatoxin M1, AFM1) retains most of AFB1 toxicity (Caloni, 2006) (International Agency for Research on  
70 Cancer, 2002); therefore, maximum tolerable levels have been established also for AFM1 in milk (European  
71 Commission, 2010). Conversely, the carry-over of AFB1 into meat of animals fed with  
72 contaminated material is controversial (Hayes, 1977) (Díaz-Zaragoza, 2014) (Hussain, 2010) and the risk for  
73 consumers associated to meat consumption seems to be negligible. Recently, the potential AFB1 carry-over  
74 into eggs in laying hens fed with contaminated crops has been investigated. Pandey and Chauhan reported  
75 on the effect of ingesting AFB1 contaminated grain on chicks (Chauhan, 2007). AFB1 residues were  
76 detected in eggs and breast muscle of AFB1-fed hens. The carry-over of AFB1 was confirmed by the works

77 of Hassan et al (Hassan, 2012) and of Herzallah (Herzallah, 2013), who also studied the combined effect of  
78 the four major aflatoxins. He found an analogous carry-over for AFB1 and the other three aflatoxins. Hassan

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86 et al observed that AFB1 residues appeared in eggs after 5 days from starting administration of  
87 contaminated feedstuffs and that AFB1 accumulated in eggs with increasing amounts found for protracted  
88 feeding with contaminated grain. Nevertheless, the amounts of aflatoxin residues found in eggs were very  
89 low in every case and varied between 0.01% (Herzallah, 2013) and 0.07% (Hassan, 2012) of the aflatoxin  
90 intake. This result could be partially explained by the fact that AFB1 is metabolized by the bird (Rawal,  
91 2010). Indeed, the metabolic transformation of AFB1 was responsible of diseases observed on hens and  
92 highlighted by the same authors. However, none of the preceding papers considered the metabolic  
93 detoxification pattern which led to the formation of hydroxylated metabolites of AFB1 (AFM1 and aflatoxin  
94 Q1) (Rawal, 2010) and authors did not investigate the occurrence of AFM1 in eggs, similarly to what is  
95 done in milk.

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96 World egg production involved over 60 millions tonnes per year from a total of approximately 6.5 billion  
97 hens and expanded by more than two per cent a year in the last decades (Nutriad). China is the world  
98 largest egg producer and is accounted for one third of the entire world production, followed by USA and  
99 India. Countries belonging to the European Union produce approximately 7.5 million tonnes of egg per year  
100 (European Commission). The demand of feed for sustain poultry production makes suspect on its quality,  
101 also because most of the ingredients used to produce poultry feed are used for human consumption. Thus,  
102 the risk that materials discarded for human consumption could be employed as feedstuffs is not negligible.  
103 Furthermore, since poultry production is relatively inexpensive and widely available and, as poultry meat  
104 and eggs are considered low-cost sources of protein, their production is strongly encouraged in developing  
105 countries, which led sometimes to not adequate housing and management of animals and feedstuffs and to  
106 increased risk of contamination (FAO, 2013).

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107 Therefore, the accessibility of rapid, cost-effective, and simple methods of analysis to detect aflatoxins in  
108 eggs would help scientists to better investigate the occurrence of these contaminants and to more  
109 adequately support conclusions on risks for human health due to consumption of eggs belonging to hens  
110 fed with aflatoxin contaminated materials. Moreover, it would allow the efficient and continuous  
111 monitoring of such contaminants to assure food security. Analytical methods to determine aflatoxins in  
112 eggs currently available are based on chromatographic techniques coupled to fluorescence or mass

134 spectrometric detection (Herzallah, 2009) (Garrido Frenich, 2011) (Capriotti, 2012). However, to ensure the  
135 rapid and cost-effective screening of large numbers of sample and the availability of analytical methods  
136 applicable in developing countries, the exploitation of immunochemical methods of analysis, which are  
137 known to address requirements of rapidity, simplicity and inexpensiveness, is advisable.

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138 This study aimed at developing a rapid and sensitive competitive enzymatic immunoassay for measuring  
139 most relevant aflatoxins in eggs. Therefore, two hapten derivatives were synthesized, with the objective of  
140 raising polyclonal antibodies able to bind the principal aflatoxin (AFB1), the main AFB1 metabolic product

141 (AFM1) and possibly the other three relevant aflatoxins (AFG1, AFB2, and AFG2). By exploiting those  
142 antibodies, two direct competitive immunoassays could be proposed: an AFB1-selective assay and a group-  
143 selective assay. This last allowed us to detect all above-mentioned mycotoxins. Moreover, aflatoxin  
144 extraction from eggs was optimized with the aim of fulfilling the same requirements of rapidity, easy  
145 operation and cost-effectiveness to allow the applicability of the whole analytical protocol as a screening  
146 method in routine monitoring of aflatoxin contamination in eggs.

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## 148 MATERIALS AND METHODS

### 149 Materials

150 Aflatoxin B1, aflatoxin M1, aflatoxin B2, aflatoxin G1, aflatoxin G2, ochratoxin A (OTA), deoxynivalenol  
151 (DON), fumonisin B1 (FB1), and zearalenone (ZEA) standard solutions were Oekanal certified solutions from  
152 Sigma Aldrich (St. Louis, MO, USA). Aflatoxin B1 and aflatoxin M1 powders were purchased from Fermentek  
153 (Jerusalem, Israel). Bovine serum albumin (BSA), N,N'-dicyclohexylcarbodiimide (DCC), N-  
154 hydroxysuccinimide (NHS), and 3,3',5,5'-tetramethylbenzidine liquid substrate (TMB) were purchased from  
155 Sigma- Aldrich (St. Louis, MO, USA). Horse-radish peroxidase (HRP) was purchased from Roche Diagnostics  
156 (Milan, Italy). Sephadex G-25 cartridges were from GE Healthcare (Milan, Italy). Dimethylformamide (DMF),  
157 methanol (HPLC grade) and all other chemicals and microtitre plates were obtained from VWR  
158 International (Milan, Italy).

### 160 Production of the hapten, hapten-protein conjugates and antibodies

165 Aflatoxin B1-O-(carboxymethyl)oxime (AFB1-cmo) and Aflatoxin M1-O-(carboxymethyl)oxime (AFM1-cmo),  
166 were synthesized from AFB1 and AFM1, respectively, as previously reported (Chu, 1977). The two haptens  
167 (Figure 1) were conjugated to BSA by the DCC/NHS ester method and used for immunization; AFB1-cmo  
168 was also conjugated to HRP to generate the labelled probe. Briefly, equimolar amounts of AFB1-cmo or  
169 AFM1-cmo, DCC and NHS were dissolved in anhydrous DMF and the mixture was incubated at 4°C  
170 temperature for 2 hours. Proper amounts of the mixture were then added to protein solutions prepared in  
171 0.13 M NaHCO<sub>3</sub>, to obtain a final molar ratio of 200:1 (AFB1-cmo or AFM1-cmo:BSA), and 10:1 (AFB1-  
172 cmo:HRP). BSA conjugates (AFB1-BSA and AFM1-BSA) were incubated overnight at room temperature,  
173 while the HRP conjugate (AFB1-HRP) was reacted for 1 hour at room temperature. Separation of conjugates  
174 from by-products and excess of reagents was carried out by gel filtration on a Sephadex G-25 cartridge  
175 (mobile phase: phosphate buffer saline).

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176 Anti-AFB1 and anti-AFM1 antibodies were produced by Davids Biotechnologie (Germany) by using their  
177 standard immunization protocol for rabbit polyclonal antibodies (Davids Biotechnologie) and sera were  
178 collected after 70 days from the first injection. The immunoglobulin fraction was obtained from antisera by  
179 ammonium sulphate precipitation and used without further purification.

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### 181 Competitive direct ELISA

182 We prepared the immunoreactive solid phase by coating wells with 150 µl of anti-AFB1 or anti-AFM1 rabbit  
183 polyclonal antibodies diluted in carbonate/bicarbonate buffer pH 9.6 (overnight at 4°C). To assure complete  
184 saturation of well surface, after washing plates with 0.05% Tween 20, we further incubated 300 µl of  
185 phosphate buffer supplied with 0.15M NaCl and 0.5% BSA (PBS@BSA) for 1 hour at room temperature,  
186 followed by washing wells with 0.05% Tween 20.

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187 The construction of calibration curves involved mixing 100 µl of AFB1-HRP (0.08 µg ml<sup>-1</sup>) in PBST@BSA and  
188 100 µl of AFB1 standards diluted in aqueous methanol (35%) at concentrations ranging from 0 to 2500 µg l<sup>-1</sup>  
189 <sup>1</sup>. After 15 minute incubation in immunoreactive wells, unbound reagents were removed by five washings  
190 with a washing solution including 0.3M NaCl and 0.05% Tween 20. Colour development was obtained by a  
191 15 min incubation with TMB (200 µl per well). The addition of 50 µl of sulphuric acid (2M) stopped colour

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197 development and allowed absorbance recording at 450 nm. For egg samples, extracts prepared as  
198 described below were directly added to wells instead of AFB1 standards. All standards were measured in  
199 duplicate, whereas samples were measured in quadruplicate.

200 Unknown sample concentrations were determined by interpolation on the calibration curve, where the  
201 signal was plotted against the log of analyte concentration. For each experiment, a calibration curve was  
202 determined by a nonlinear regression analysis of the data using the four-parameter logistic equation.

203

#### 204 **Cross-reactivity study**

205 We prepared calibration curves for several mycotoxins, by employing the same protocol described above,  
206 except from the concentration range used, which depended on the investigated mycotoxin. We used 0 - 2.5  
207  $\mu\text{g l}^{-1}$  for AFB1; 0 - 10  $\mu\text{g l}^{-1}$  for AFG1, AFB2, and AFG2; and 0 - 100  $\mu\text{g l}^{-1}$  for OTA, DON, FB1, and ZEA,  
208 respectively.

209 Relative cross-reactivity was calculated as follows:

$$210 \text{ CR\%} = (\text{IC}_{50} \text{ AFB1} / \text{IC}_{50} \text{ mycotoxin}) * 100$$

211 where  $\text{IC}_{50}$  is the mycotoxin concentration which cause 50% inhibition of the maximum observed signal.

212 The estimated limit of detection for aflatoxin (except from AFB1) was derived from CR% by dividing LOD  
213 calculated for AFB1 by the cross-reactivity as follows:

$$214 \text{ Estimated mycotoxin LOD} = (\text{LOD AFB1} / \text{CR\% mycotoxin}) * 100$$

215

#### 216 **Samples and sample preparation**

217 Egg samples were purchased in large stores (Large-scale distribution, LSdis) or directly in farms of small-  
218 scale producers (Farm) of the North-West of Italy during the period January-March 2014.

219 Two samples that did not show any detectable residues of aflatoxins were taken as the blank for the  
220 optimization of the extraction protocol and for recovery experiments. Fortified samples were prepared by  
221 adding 0.5, 2.0, and 10.0  $\mu\text{g kg}^{-1}$  or 1.0, 4.0, and 10.0  $\mu\text{g kg}^{-1}$  of AFB1, respectively, to the egg before  
222 performing the extraction.



223 Egg yolk was manually separated and gently mixed before extractions. To optimize extraction of aflatoxins  
224 from egg we weighed 1 g of the homogenized sample and mixed it with 5 ml of various extraction media: 1)  
225 water to which 0.05% Tween 20 had been added (0.05% Tween 20); 2) water : methanol 30:70 (methanol  
226 70%); 3) water : methanol 70:30, in which 0.3M NaCl had been added to water (methanol 70%/0.3M NaCl);  
227 and 4) water : methanol 70:30 followed by the addition of hexane to remove fatty components. After 2  
228 minutes of vigorous stirring at room temperature, samples were centrifuged at 3200 x g to reduce foam  
229 and to remove denatured proteins. Supernatants were diluted 1+1 with water and analysed by the direct  
230 competitive ELISA. Each sub-sample was extracted in duplicate and analysed in quadruplicate.

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231 The optimal protocol involved extraction with aqueous methanol (70%) followed by defatting with hexane.  
232 Briefly, we recovered the supernatant after the extraction described above and added 1 ml of hexane. We  
233 vigorously stirred the mixture for 2 minutes again, separated the upper organic layer by centrifugation (5  
234 minutes at 3200 x g), and diluted the underlying layer 1+1 with water before submitting it to analysis.

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**Spostato (inserimento) [1]**

**Eliminato:** Production of polyclonal antibodies¶

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**Eliminato:** the IC<sub>50</sub> (antibody dilution which determined the 50% of inhibition of the maximum observed signal) were measured to be 1:160,000 and 1:120,000 for the two rabbits immunized with AFB1-BSA, and 1:75,000 and 1:63,000 for the two rabbits immunized with AFM1-BSA.

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**Eliminato:** and similar features (Figure 2). Arbitrarily, anti-AFB1-R1 antiserum was used for this study.

**Eliminato:** Among the two anti-AFM1 antisera,

**Eliminato:** anti-AFM1-R1 antiserum demonstrated better binding properties towards the analyte (Figure 2) and thus was chosen for the study.

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**Spostato in su [1]: Competitive direct ELISAs¶**

**Eliminato:** in the attempt of achieving sensitivity and rapidity

**Eliminato:** For sensitivity study, we compared the IC<sub>50</sub> parameter obtained from standard curves carried out in the evaluated experimental conditions, i.e. the analyte concentration causing 50% inhibition of the signal recorded in the absence of the analyte itself.

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## 236 RESULTS AND DISCUSSION

### 237 Competitive direct ELISAs

238 Four antisera were produced by immunizing two rabbits with AFB1-BSA and two with AFM1-BSA,  
239 respectively. These were tested by a non-competitive indirect ELISA, by following the protocol described in  
240 the experimental section, except for the facts that antibodies were coated at increasing dilutions and that  
241 the analyte was absent. All four generated antisera show high titres. Their performance in competitive  
242 conditions were also tested by determining the rate between the signal obtain from each antiserum diluted  
243 at its IC<sub>50</sub> value in the presence of AFB1 (10 µg l<sup>-1</sup>) and in the absence of AFB1 (0 µg l<sup>-1</sup>). Both anti-AFB1  
244 antisera and one of the anti-AFM1 antisera exhibited high binding properties towards the analyte (AFB1).  
245 One anti-AFB1 antiserum and the anti-AFM1 with high binding properties were used for the study.

246  
247 Competition experiments were carried out under various combinations of antibody dilutions, AFB1-HRP  
248 concentrations and times of incubation to set a sensitive and rapid assay. Since aflatoxin extraction from  
249 food materials involves the use of aqueous methanol in most cases (Stroka, 1999) (Reiter, 2009), the effect

285 of the solvent on assay performance was also evaluated. We found that methanol interference was  
286 negligible for amounts up to 20%, thus we established to dilute AFB1 standards in 35% methanol for  
287 calibration. In this way, extracts, which would contain 70% methanol, could easily match calibrators by  
288 being diluted 1+1 with water. In the meantime, standard and extracts would be further diluted 1+1 in wells  
289 by mixing them with the AFB1-HRP solution which permitted us to reach overall methanol content below  
290 20% during the assay.

291 Preliminary, we optimized two systems: a homologous assay, which employed the anti-AFB1 antibody and  
292 the AFB1-HRP as the probe, and a heterologous assay, which employed the anti-AFM1 antibody and the  
293 same AFB1-HRP probe, since heterology is known to promote greater sensitivity (Holthues, 2005) (Z. Wang,  
294 2013). Figures of merits of the two optimized assays, carried out as described in the experimental section,  
295 are summarized in Table 1 and typical inhibition curves obtained under optimized conditions for the two  
296 systems are shown in Figure 3. Both systems were highly sensitive and very rapid, provided that results  
297 could be achieved in 30 min. Assay characteristics are comparable in terms of detectability and dynamic  
298 range.

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### 300 Selectivity

301 Since we did not know if the supposed ingested aflatoxin would be preserved as it or metabolized by the  
302 bird before transference to the egg, we aimed at developing an assay which would cross-react with the four  
303 major aflatoxins and also with the metabolic product, aflatoxin M1.

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304 We studied the selectivity of the two developed ELISAs by measuring their cross-reactivity towards  
305 mentioned aflatoxins (AFB1, AFM1, AFG1, AFB2, and AFG2) and towards other mycotoxins, whit chemical  
306 structures completely unrelated to those of aflatoxins (DON, FB1, OTA, and ZEA). The interference with  
307 both assays was negligible for all unrelated mycotoxins. These did not cause any inhibition of the binding  
308 between antibodies and the AFB1-HRP probe at levels up to 100  $\mu\text{g l}^{-1}$ .

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309 Instead, we observed a dissimilar binding capacity of the two ELISAs when aflatoxins were applied, which  
310 was imputable to the antibodies used. The system based on the anti-AFB1 antiserum showed a selective  
311 pattern, in which only AFG1 and AFB2 were detected in some extent, besides AFB1 (Figure 4). Otherwise,

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319 the assay based on the anti-AFM1 antiserum behaved as a non-selective system, since AFB1, AFM1 and  
320 AFG1 inhibited the binding of AFB1-HRP probe to the antibodies quite similarly, and also AFB2 and AFG2  
321 were effective at competing with the probe for antibody binding (Figure 3b and 4). Therefore, we assumed  
322 the assay based on the anti-AFB1 antiserum as applicable for the selective determination of AFB1 (AFB1-  
323 assay), whereas the assay exploiting the anti-AFM1 antiserum was considered as being able to detect most  
324 aflatoxins and thus, as a group-selective assay. Since the purpose of the work, the system based on anti-  
325 AFM1 antiserum was further characterized and applied to aflatoxin determination in eggs.

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### 327 **AFB1 extraction from egg**

328 According to the literature, the most popular protocols for extracting aflatoxins from food and feed to be  
329 measured by immunoenzymatic methods involve aqueous methanol with percentages varying from 60 to  
330 80% and typically 70% (Stroka, 1999) (Reiter, 2009). Depending on the sample nature, high amounts of  
331 sodium chloride and/or a step aimed at removing fats could be introduced (Garden, 2001) (Shadbad,  
332 2012). However, also aqueous extractants have been reported to be effective in the extraction of aflatoxins  
333 from cereals (Maragos, 2008) (Anfossi, 2011). Therefore, we compared recovery of AFB1 from an artificially  
334 contaminated egg sample, when extracted by various media including: water, aqueous methanol (70%),  
335 aqueous methanol with sodium chloride added, and aqueous methanol followed by defatting with hexane.  
336 Results of tests on the egg fortified at three AFB1 levels: 0.5 (low), 2.0 (medium), and 10.0  $\mu\text{g kg}^{-1}$  (high) are  
337 summarised in Figure 5. The aqueous extraction medium was inadequate (recoveries comprises between  
338 15 and 24%) while 70% methanol confirmed its superior quality. The addition of the salt slightly impaired  
339 performance, while appending a step aimed at removing fats from extracts contributed to achieve  
340 quantitative results, most likely because fats interfered in the immunoassay determining underestimation.

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### 342 **Analytical validation of the group-selective immunoassay for measuring aflatoxins in eggs**

343 The LOD of the method was calculated by interpolation on the AFB1 standard curve as the analyte  
344 concentration corresponding to the mean signal of the zero standard (obtained by averaging the signal of  
345 eight replicate sets) minus three times its standard deviation and it was 0.03  $\mu\text{g J}^{-1}$  for both the AFB1-assay

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349 and the group-selective assay (Table 1). Adjusted for dilution due to extraction, the limits of detection of  
350 the two formats compared to egg samples were 0.3  $\mu\text{g kg}^{-1}$  for AFB1. The group-selective assay allowed us  
351 to measure AFG1 and AFM1 at the same level as AFB1 (LOD 0.3  $\mu\text{g kg}^{-1}$ ) and AFB2 and AFG2 with a LOD of 3  
352  $\mu\text{g kg}^{-1}$ , which are in the range of limits imposed by the European legislation on various food, except for  
353 AFM1 in milk (European Commission, 2010).

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354 To evaluate the accuracy of the method, two egg samples were fortified with AFB1 (concentrations of 1.0,  
355 4.0, and 10  $\mu\text{g kg}^{-1}$ ), extracted and analysed by the group-selective assay. Results are summarized in Table

356 2. Recovery values ranged from 84 to 100%, thus indicating a good accuracy of the assay when applied to

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357 egg samples. The precision of the method was determined by extracting and analysing replicates of

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358 artificially contaminated egg samples, which were fortified with AFB1 at three levels: 0.5, 2.0, and 10.0  $\mu\text{g}$   
359  $\text{kg}^{-1}$ . The assay was carried out in eight replicates on the day for the evaluation of within-assay precision

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360 and on four different days for the evaluation of the between-assay precision (Table 3). The values of RSD%

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361 were calculated at each nominal concentration level and ranged from 8 to 20%, which fulfilled FDA  
362 requirements for the validation of bioanalytical methods according to FDA guidance (Anfossi, 2009).

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363 These results proved that the developed assay is suitable as a first level screening method for the detection  
364 of aflatoxins in eggs, with good accuracy and precision.

**Eliminato:** The assay is sensitive, also because sample extracts need a very limited dilution before being analysed, thus LOD in eggs is 0.3  $\mu\text{g kg}^{-1}$  for principal aflatoxins (AFB1, AFG1, and AFM1) and 3  $\mu\text{g kg}^{-1}$  for AFB2 and AFG2. In addition, the method is simple, very rapid (the extraction requires 14 minutes, and the assay is completed in 30 minutes), and we could decide to measure the most relevant aflatoxin by the AFB1-selective assay or, alternatively, to detect generically chemical structures structurally related to AFB1 through the group-selective assay. The last allowed us to determine several aflatoxins and could potentially apply for the detection of AFB1, AFG1, AFG2 and AFG2 contaminated samples due to carry-over, as for the detection of samples containing the most relevant AFB1 metabolite (AFM1).

365 Finally, we collected a total of 50 samples, belonging to the large distribution and to small farms located in  
366 the North West of Italy, during the period of January-March 2014. No positive samples were found in the  
367 market according to analysis through the developed group-selective ELISA method.

368

## 369 CONCLUSIONS

370 The occurrence of aflatoxin contamination in cereals poses severe risk to consumers not only through their  
371 consumption, but also through entering the food chain. This has been widely demonstrated in the case of  
372 milk belonging to dairy animals fed with aflatoxin contaminated crops. Aflatoxin carry-over in eggs has also  
373 been reported recently. Therefore, the development of analytical methods aimed at measuring such  
374 contaminants in eggs would help assuring food safety within the whole food chain. For this purpose, we  
375 developed an enzymatic assay for monitoring aflatoxins in eggs and established a fast, simple and effective

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402 extraction protocol, based on the use of aqueous methanol as the extractant and of hexane to remove fatty  
403 components of the matrix.

404 The developed assay is sensitive, also because sample extracts need a very limited dilution before being  
405 analysed, thus LOD in eggs are  $0.3 \mu\text{g kg}^{-1}$  for principal aflatoxins (AFB1, AFG1, and AFM1) and  $3 \mu\text{g kg}^{-1}$  for  
406 AFB2 and AFG2. In addition, the method is simple, very rapid (the extraction requires 14 minutes, and the  
407 assay is completed in 30 minutes), and we could decide to measure the most relevant aflatoxin by the  
408 AFB1-selective assay or, alternatively, to detect generically chemical structures structurally related to AFB1  
409 through the group-selective assay. The last allowed us to determine several aflatoxins and could potentially  
410 apply for the detection of AFB1, AFG1, AFG2 and AFG2 contaminated samples due to carry-over, as for the  
411 detection of samples containing the most relevant AFB1 metabolite (AFM1).

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491 **FIGURE CAPTIONS**

492

493 **Figure 1:** Structure of the five principal aflatoxins, the hapten derivatives used for immunization and for  
494 preparation of the enzymatic tracer, and the mycotoxins used in the cross-reactivity study.

495

496 **Figure 2:** Characterization of polyclonal antibodies generated by immunizing two rabbits with AFB1-BSA  
497 (anti-AFB1-R1 and anti-AFB1-R2) and two with AFM1-BSA (anti-AFM1-R1 and anti-AFM1-R2). Signals  
498 recorded by the competitive direct ELISA when AFB1 was added at 0 (black column) and 10  $\mu\text{g l}^{-1}$  (grey  
499 column).

500

501 **Figure 3:** Typical inhibition curves obtained under optimized conditions for the two ELISA systems  
502 developed: a. AFB1 standard curve in the selective assay (signal vs. AFB1 concentration); b. standard curves  
503 for the five major aflatoxins in the groupe-selective assay ( $\circ$  AFB1,  $\triangle$  AFM1,  $\square$  AFG1,  $\nabla$  AFB2,  $\diamond$  AFG2).  
504 For comparison, the B/B0 vs. mycotoxin concentration is shown, where B is the signal observed for the  
505 mycotoxin concentration and B0 is the signal of the blank.

506

507 **Figure 4:** Cross-reactivity of aflatoxins determined by the two ELISAs towards major aflatoxins.

508

509 **Figure 5:** Extraction of AFB1 from an artificially contaminated egg sample by varying the extraction  
510 medium. Egg sample was fortified at three AFB1 concentration levels: 0.5  $\mu\text{g l}^{-1}$  (low, black), 2  $\mu\text{g l}^{-1}$   
511 (medium, grey), and 10  $\mu\text{g l}^{-1}$  (high, white).

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