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1 **Peptide binding to ochratoxin A mycotoxin: a new approach in conception of biosensors.**

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10 **Abstract**

11 Ochratoxin A (OTA) is a widespread and abundant natural carcinogenic mycotoxin produced  
12 by several species of *Aspergillus* and *Penicillium* fungi. Due to the ubiquitous presence of  
13 these fungi in food and potential risk for human health, a rapid and sensitive *in vitro* detection  
14 assay is required. Analytical methods for OTA detection/identification are generally based on  
15 liquid-liquid extraction, clean-up using an immunoaffinity column (IAC), and identification  
16 by reversed-phase high pressure liquid chromatography with fluorescence detection (HPLC-  
17 FLD). However, IACs are costly and have a short lifespan. Therefore, an interesting approach  
18 would appear to be the design and chemical synthesis of a mimotope peptide simulating  
19 mycotoxin-specific antibodies. We have developed a promising alternative method that is  
20 based on the use of peptides which are able to bind to specific chemical functions and/or  
21 molecular structures. Accordingly, a number of peptides (derived from the structures of major  
22 redox proteins) were selected and produced by chemical solid phase syntheses. The ability of  
23 such peptides to bind to ochratoxin A was evaluated by HPLC. The peptide NF04  
24 (structurally derived from an oxidoreductase enzyme), which was found to be the sole  
25 potentially reactive compound among tested molecules, was further evaluated in a peptide-based  
26 enzyme-linked immunosorbent assay (peptide-based ELISA), thus confirming its specific  
27 interaction with ochratoxin A.

28 **Keywords:** peptide binding, peptide-based assay, ochratoxin A, mycotoxin

29

30 **1. Introduction**

31 Ochratoxin A, also referred to as OTA, is a coumarinic mycotoxin produced by several fungi  
32 species from *Aspergillus* (e.g., *A. ochraceus*) and *Penicillium* (e.g., *P. verrucosum*) genera  
33 under different environmental conditions (Scott et al., 1997; Brera et al., 2008). It is a  
34 mycotoxin that has been identified as a contaminant in grains, cereals, beans, coffee, dried  
35 fruits and wine (Zimmerli et al., 1995, Varga et al., 2006; Blessa et al., 2006). OTA is known  
36 to have nephrotoxic, immunotoxic, teratogenic and carcinogenic effects (O'Brien et al., 2005,  
37 Pfohl-Leskowicz et al., 2002, Smith et al., 1995). Wine contamination by OTA has been  
38 described and largely reviewed by several authors (Varga et al., 2006; Blessa et al., 2006).  
39 This beverage is widely consumed and represents a major source of daily OTA intake for the  
40 population (Jorgensen et al., 2005). Thus, regulatory limits for OTA exist in many countries,  
41 especially in Europe where maximum limits for OTA in wine, grape juices and grape  
42 beverages, have been fixed at  $2 \mu\text{g l}^{-1}$  (European Union, 2005, 2010). Nowadays, the most  
43 widely used quality control process relies on an immunoaffinity column (IAC), followed by  
44 reversed-phase high pressure liquid chromatography using fluorescence detection (HPLC-  
45 FLD) (Visconti et al., 1999, Aresta et al., 2006). Because this mycotoxin is largely  
46 represented in food, availability of rapid, reliable and sensitive analytical methods for the  
47 detection of OTA is required to protect consumers' health. Despite the fact that the IAC  
48 procedure is rather simple, sensitive and quite reproducible, IACs are unfortunately too costly,  
49 together with short shelf lives. In the last decade, several groups attempted to develop  
50 appropriate alternative assays to improve rapidity and sensitivity, combined with cost  
51 reduction. Such methods rely on immunoassays, test strips and biosensors. First, a  
52 competitive ELISA kit has been used widely in recent years for the detection of OTA.  
53 ELISAs for ochratoxin content analyses have been reported in barley (Morgan et al., 1983).  
54 The assay sensitivity for detection of OTA in barley samples was circa  $5 \mu\text{g kg}^{-1}$   
55 (Ramakrishna et al., 1990). Angelini et al., (2008) compared performance of four extraction  
56 procedures and three commercial ELISA kits for OTA in grapes. Sometimes, IAC are used to  
57 concentrate OTA. The advantage of using IAC after the extraction procedure was the  
58 excellent detection limit, which was between  $0.06$  and  $0.0075 \mu\text{g l}^{-1}$ . This detection limit  
59 depends on the ELISA kit used. Second, the test strip, also called lateral flow device or  
60 immunochromatographic strip (ICS) test, is based on a membrane loaded with immobilized  
61 antibodies. They are of simple use and give faster results (2 to 15 min). Test strips are semi-  
62 quantitative with different visual limits of detection (LOD) in function of the nature of sample  
63 (Krska et al., 2009; Shim et al., 2009). Initially, the LOD was set at ca.  $500 \mu\text{g l}^{-1}$  of OTA  
64 (Cho et al., 2005; Rusanova et al., 2009), whereas, nowadays, the cutoff level dropped down

65 to  $1 \mu\text{g l}^{-1}$ , which corresponds to the lower limit tolerated by the Food and Drugs  
66 Administration. Third, with regard to biosensors, their characteristics depend on the nature of  
67 the bioreceptor and the physical transducer. Antibodies, which show high selectivity and  
68 affinity towards mycotoxins, have been widely used to set up a variety of immunosensors  
69 (e.g. electrochemical, impedimetric or conductimetric immunosensors) against mycotoxins  
70 like OTA (Pietro-Simon et al., 2008; Liu et al., 2009; Alacon et al., 2006; Radi et al., 2008  
71 and 2009).

72 The well-known favorable molecular recognition characteristics of an antibody (in  
73 terms of affinity and selectivity) are counterbalanced by the unfavorable use of different  
74 matrix samples or experimental conditions of assay (e.g. denaturation of antibodies in organic  
75 solvents). To overcome these drawbacks, several strategies have been followed such as  
76 development of new synthetic systems that mimic the recognition properties of antibodies.  
77 Indeed, many efforts have been made to substitute OTA antibodies by DNA aptamers (Cruz-  
78 Aguado et al., 2008a, 2008b), molecularly imprinted polymers (MIP) (Ali et al., 2010; Yu et  
79 al., 2010) and phage display libraries (Giraudi et al., 2007). The isolation of oligonucleotide  
80 sequences (DNA aptamers) and synthetic receptor(s) (MIP) that recognize this class of target  
81 molecules have some advantages compared to antibodies. They can be generated easily and  
82 are stable at different pH values and/or at high temperatures. **Among these approaches, based  
83 on synthetic systems, none of them reach affinity for OTA that is compatible with the  
84 detection limits fixed in wine by the European Commission ( $2.0 \mu\text{g l}^{-1}$ ) or the rest of the  
85 world ( $1.0 \mu\text{g l}^{-1}$ ).** The first hexapeptide selected using phage display libraries exhibits an  
86 affinity of ca.  $3.4 \times 10^4 \text{ M}^{-1}$  towards OTA (Giraudi et al., 2007). Although moderate, such a  
87 peptide affinity can be potentially increased by some structure-activity relationship studies.  
88 Peptide-based detection assays in general are commercially available and most frequently  
89 used in the biomedical field rather than environmental sciences which is of concern in this  
90 study. For examples, peptides are used in various fields, from diagnosis of HIV infection  
91 (Alcaro et al., 2003; Ravanshad et al., 2006, Gerasimov 2010) to detection of potential  
92 sensitizing compounds (Gerberik et al., 2004).

93 Apart from the previously described techniques used for OTA quantification, we describe in  
94 this work for the first time a novel approach based on the identification of new peptides (not  
95 based on phage display analyses) which exhibit significant affinities towards OTA. HPLC  
96 was used as an analytical method to select the most potent peptide interacting with OTA in a  
97 binding assay. Identification of such a peptide is important and allowed us to analyze some

98 red wine samples that were previously supplemented with OTA in a peptide-based enzyme-  
99 linked immunosorbent assay (peptide-based ELISA).

## 100 2. Material and methods

### 101 2.1. Materials

102  $N^\alpha$ -fluorenyl-9-methyloxycarbonyl (Fmoc)-L-amino acids, Fmoc-amide rink resin, and  
103 reagents used for peptide synthesis were obtained from Iris Biotech (Germany). Solvents were  
104 analytical grade products from Carlo-Erba (France).

105

### 106 2.2. Chemicals

107 OTA was obtained from Sigma-Aldrich (France). A solution was prepared in methanol at 1  
108  $\text{mg ml}^{-1}$ . PEG 8000 (Polyethylene Glycol) and PVPP were obtained from Promega (France).  
109 Luminol was obtained from Pierce (France).

110

### 111 2.3. Solid-Phase Peptide Synthesis

112 The peptides (NF01, NF02, NF03, NF04, Chim1, pep01 and pep02) were produced by  
113 chemical synthesis using a peptide synthesizer (Model 433A, Applied Biosystems Inc.). **The**  
114 **amino acid sequence of the most reactive peptide, i.e. NFO4, is provided in Fig. 2B. All**  
115 **peptide sequences are described in European patent n° 12305269.8 (deposited by Tournoux**  
116 **Biotech on March 5<sup>th</sup> 2012).** Peptide chains were assembled stepwise on 0.25 mmol of Fmoc-  
117 amide resin (1% cross-linked; 0.65 mmol of amino group/g) using 1 mmol of  $N^\alpha$ -(9-  
118 fluorenyl)methyloxycarbonyl (Fmoc) L-amino acid derivatives. Side chain-protecting groups  
119 for trifunctional residues were: trityl for cysteine, and asparagine; *t*-butyl for tyrosine,  
120 glutamate and aspartate; 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl for arginine; and  
121 *t*-butyloxycarbonyl for lysine.  $N^\alpha$ -amino groups were deprotected by successively treating  
122 with 18 and 20% (v/v) piperidine/*N*-methylpyrrolidone for 3 and 8 min, respectively. After  
123 three washes with *N*-methylpyrrolidone, the Fmoc-amino acid derivatives were coupled (20  
124 min) as their hydroxybenzotriazole active esters in *N*-methylpyrrolidone (4-fold excess). After  
125 peptides were assembled, and removal of N-terminal Fmoc groups, the peptide resins (ca. 1.5  
126 g) were treated under stirring for 2.5 h at 25°C with mixtures of trifluoroacetic  
127 acid/ $\text{H}_2\text{O}$ /thioanisole/ethanedithiol (73:11:11:5, v/v) in the presence of crystalline phenol (2.1  
128 g) in final volumes of 30 ml per gram of peptide resins. The peptide mixtures were filtered,  
129 precipitated and washed twice with cold diethyloxide. The crude peptides were pelleted by  
130 centrifugation ( $3,200 \times g$ ; 10 min). They were then dissolved in  $\text{H}_2\text{O}$  and freeze dried. The

131 crude peptides were purified to homogeneity by reversed-phase high pressure liquid  
132 chromatography (HPLC) (C<sub>18</sub> Aquapore ODS, 20 μm, 250 × 10 mm; PerkinElmer Life  
133 Sciences) by means of a 60-min linear gradient of 0.08% (v/v) trifluoroacetic acid/H<sub>2</sub>O  
134 (buffer A) with 0 to 40% of 0.1% (v/v) trifluoroacetic acid/acetonitrile (buffer B), at a flow  
135 rate of 4 ml/min (λ = 230 nm). The purity and identity of each peptide were assessed by: (i)  
136 analytical C<sub>18</sub> reversed-phase HPLC (C<sub>18</sub> Lichrospher 5 μm, 4 × 200 mm; Merck) using a 60  
137 min linear gradient of buffer A with 0-60% of buffer B, at a flow rate of 1 ml/min; and (ii)  
138 molecular mass determination by matrix-assisted laser desorption ionization-time of flight  
139 (MALDI-TOF) *spectrometry (Voyager DE-RP, Perceptive Biosystems Inc.)*.

140

#### 141 *2.4. HPLC-based peptide binding assays*

142 Eighty microlitres of a peptide (NF01, NF02, NF03, NF04, Chim1, pep01 or pep02) at a  
143 concentration of 1.25 mM in 0.1 M Tris-HCl buffer, pH 8.3, were tested with 10 μl of OTA  
144 solution at 0.1 M in acetonitrile, supplemented with 70 μl of 0.1 M Tris-HCl buffer (pH 8.3)  
145 and 40 μl acetonitrile. The mixture of peptide and OTA was incubated for 4 h in the dark, at a  
146 temperature of 30°C. The reaction medium (200 μl) was then analyzed by C<sub>18</sub> reversed-phase  
147 HPLC (C<sub>18</sub> Aquapore ODS, 20 μm, 250 × 10 mm; PerkinElmer Life Sciences) by means of a  
148 40-min linear gradient of 0.08% (v/v) trifluoroacetic acid/H<sub>2</sub>O (buffer A) with 0 to 60% of  
149 0.1% (v/v) trifluoroacetic acid/acetonitrile (buffer B), at a flow rate of 1 ml/min (λ = 230 nm).  
150 Peptide reactivity with OTA was finally assessed by comparing the peak areas corresponding  
151 to free peptide (unreactive peptide) between the test sample of peptide/OTA, and a reference  
152 sample of peptide alone (without OTA). It is worth mentioning that results obtained with the  
153 reference samples (peptides alone) are similar to those obtained with peptides incubated with  
154 irrelevant, unreactive products (data not shown). The identity of free peptides and  
155 peptide/OTA complexes was verified by MALDI-TOF mass spectrometry. Binding assays  
156 were performed in triplicate.

157

#### 158 *2.5. Peptide-based competitive enzyme-linked immunosorbent assay (peptide-based* 159 *competitive ELISA)*

160 Polystyrene white microtiter plate wells (Maxisorb LumiNunc, ThermoScientific, USA),  
161 coated with the synthetic peptide NFO4 at an optimized concentration of 5 μg/100 μl in  
162 carbonate buffer (15 mM Na<sub>2</sub>CO<sub>3</sub>, 35 mM NaHCO<sub>3</sub>, 0.2 g/L NaN<sub>3</sub>, pH 9.6) were incubated at  
163 37°C for 3 h. Non-specific binding sites of the peptide-coated wells were blocked with 5%

164 nonfat dry milk in PBS containing 0.1% Triton X-100 (milk buffer) at room temperature (RT)  
165 for 3 h before performing the test. Fifty  $\mu\text{l}$  of OTA-HRP (horseradish peroxidase) were added  
166 in each well combined with 50  $\mu\text{l}$  of phosphate buffer saline or red wine sample supplemented  
167 with unlabeled OTA. The reaction was left for 30 min at RT. After washing unbound OTA,  
168 40  $\mu\text{l}$  of luminol (Pierce, France) substrate was added in each well. After 5 min of enzymatic  
169 reaction, **light emission signals** ( $\lambda_{\text{max}} = 425 \text{ nm}$ ) were analyzed using an automated  
170 microplate luminescence reader (Berthold, France). **Light intensity** was expressed in Relative  
171 Luminescent Unit (RLU). The result obtained is inversely proportional to the concentration of  
172 unlabeled OTA. During each test, nonspecific binding (negative control) was determined by  
173 using an incubation mixture (OTA-HRP) in which the peptide NFO4 was replaced by 100  $\mu\text{L}$   
174 of carbonate buffer. All the samples were tested in triplicate and the mean of **the peak light**  
175 **emission** was taken as the final **light signal** value.

176

## 177 2.6. Calculation methods

178 In order to evaluate the peptide-based competitive assay, a calibration curve was set up by  
179 using solutions containing well-defined concentrations of OTA. In that direct competitive  
180 peptide-based ELISA, results are expressed in  $B/B_0$  dose logarithmic function. B and  $B_0$   
181 represent the enzyme-bound activity measured in the presence or absence of competitor,  
182 respectively. The standard curve was traced by plotting standard concentrations on x-axis  
183 (logarithmic scale) and percentage of maximal binding (express in % of  $B/B_0$ ) on y-axis ( $B /$   
184  $B_0 = f(\log [\text{OTA}])$ ). The binding values are obtained by dividing the **light intensity** of each  
185 testing well B (the luminescence measured when OTA-HRP and unlabeled OTA are in  
186 competition with NFO4 peptide) by the **light intensity** of the positive control well  $B_0$   
187 (maximum luminescence obtained with OTA-HRP). This method allows the comparison of  
188 results between assays performed on different plates or different days. While the absolute  
189 **light emission** may differ from plate to plate or day to day, the percentage of  $B/B_0$  values  
190 should be reasonably consistent from one plate to the next. All measurements were made in  
191 triplicate. The minimum detectable concentration (MDC) was taken as the concentration of  
192 competitor (unlabeled OTA) inducing a significant decrease in  $B_0$ . The effect of complex  
193 matrix was established by testing a red wine sample.

194

## 195 2.7. Specificity measurements

196 The specificity of the peptide immunoassay described previously was controlled by testing its  
197 capacity to detect or not ochratoxin B (OTB), another mycotoxin structurally related to OTA.  
198 Results are expressed as percentage of cross-reactivity, defined as the ratio (%) of the  
199 concentration of OTA and OTB compounds at 50% B/Bo. Cross-reactivity measurement was  
200 carried out in triplicates.

201

#### 202 2.8. Preparation of matrix samples for peptide EIA: wine pretreatment

203 In order to study matrix-associated effects, a study with red wine was carried out. A sample of  
204 10 ml of wine supplemented (or not) with OTA (1.25 to 15  $\mu\text{g l}^{-1}$ ) has been diluted with 10  
205 mL of PEG8000 1% -  $\text{NaHCO}_3$  5% solution. This mixture has been incubated for 30 min at  
206 RT on a rocker. Afterwards, it was centrifuged at 8000 rpm for 15 min. The whole sample is  
207 filtered before analysis with the peptide-based enzyme-linked immunosorbent assay.

208

### 209 3. Results and discussion

210

#### 210 3.1. Rationale of the study

211 The mycotoxin OTA from *Aspergillus* (e.g., *A. ochraceus*) and *Penicillium* (e.g., *P.*  
212 *verrucosum*) genera is a complex organic compound that contains several functional groups,  
213 including carbonyl (ester:  $\text{R}^1\text{COOR}^2$ , and amide:  $\text{R}^1\text{CONHR}^2\text{R}^3$ ) and phenol (i.e.  $\phi\text{-OH}$ )  
214 moieties (Fig. 1A). We designed and chemically produce a number of peptides (European  
215 patent deposit n°12305269.8, 2012) derived from specific regions of redox proteins (e.g.  
216 oxidoreductase) and ABC transporters that potentially react -in an HPLC-based binding assay-  
217 with more or less complex molecules containing such functional group(s), i.e carbonyl and/or  
218 phenol (Table 1a and b). The carbonyl group (i.e  $\text{C=O}$ ) is shared by several types of organic  
219 compounds and comprises ketone, aldehyde, ester, amide, carboxylic acid, acid anhydride,  
220 enone and acyl halide. We evaluated whether or not these selected peptides would interact  
221 with OTA. As shown in Table 2, three peptides (NFO2, NFO3 and NFO4), with related  
222 molecular structures (up to 83% sequence identity) derived from human NADH-FMN  
223 oxidoreductase significantly interacted with the mycotoxin. The experimental molecular  
224 masses, as determined by MALDI-TOF mass spectrometry, were  $(\text{M}+\text{H})^+$ : 1793.17 Da  
225 (NFO2), 1722.15 Da (NFO3) and 1598.99 Da (NFO4), consistent with their calculated  
226 molecular structures. Among reactive peptides, NFO4 was the most potent compound, with  
227 70% binding to OTA in our experimental conditions of binding assay. NFO4 amino acid  
228 sequence is provided in Fig. 1B. Fig. 2A shows binding assays with representative HPLC  
229 profiles of three reaction media corresponding to NFO4 incubated for 4 h at 30°C with lactic



230 acid as negative control (left panel), hydroquinone as positive control (center panel) and OTA  
231 (right panel). Interaction of NFO4 with hydroquinone or OTA is highlighted by the  
232 disappearance (hydroquinone) or decrease (OTA) of peak area corresponding to free-unbound  
233 NFO4. HPLC profiles showing binding of other peptides to OTA are also shown for  
234 comparison (Fig. 2B). For example, NFO1 and Chim1 showed binding inferior to 10%, while  
235 NFO2 showed binding at 35%.

236 Using NFO4, the threshold of OTA detection was found to be in the same concentration range  
237 as the one requested by the European commission regulation ( $2 \mu\text{g l}^{-1}$  OTA). Although the  
238 potency of OTA detection by NFO4 is actually moderate, one can anticipate that optimizing  
239 both NFO4 structure (in a structure-activity relationship study) and the experimental  
240 conditions of binding assay could improve sensitivity of peptide-based detection, and yield to  
241 the desired mycotoxin detection range in wine. Overall, experimental data obtained strongly  
242 suggest that, basically, a peptide-based detection assay of OTA might be a promising  
243 approach.

244

### 245 3.2. Peptide-based competitive enzyme-linked immunosorbent assay

246 Competitive ELISAs are most commonly used to measure various molecules including lipids,  
247 hormones, and small peptides if they are present in high enough concentrations. In this study,  
248 this type of assay is based on the competition between the analyte of interest, OTA, and an  
249 enzyme horseradish peroxidase-conjugated version of the same analyte (referred to as the  
250 tracer, OTA-HRP) for a limited number of specific peptide NFO4 binding sites (Fig. 3). The  
251 concentration of OTA-HRP is held constant in all wells while the concentration of OTA  
252 varies from well-to-well ( $0 \mu\text{g l}^{-1}$ ,  $1.25 \mu\text{g l}^{-1}$ ,  $2 \mu\text{g l}^{-1}$ ,  $2.5 \mu\text{g l}^{-1}$ ,  $5 \mu\text{g l}^{-1}$ ,  $10 \mu\text{g l}^{-1}$  and  $15$   
253  $\mu\text{g l}^{-1}$ ). As a result, the amount of tracer that can bind to the peptide NFO4 will be inversely  
254 proportional to the amount of analyte in the well – the presence of more analyte means less  
255 tracer will be able to bind to the specific peptide.

256 The standard curves obtained for peptide-based competitive ELISA in PBS are shown (Fig.  
257 4A). The exponential curve fit for the standard OTA in PBS gives a clear graphical  
258 representation of how the competition proceeds. Inhibition starts at  $1.25 \mu\text{g l}^{-1}$  and reaches a  
259 maximum at  $10 \mu\text{g l}^{-1}$ . Inhibition is complete which is expected since the tracer is also OTA-  
260 based. Half-inhibition occurs at a value of  $3.2 \mu\text{g l}^{-1}$ , which should grossly correspond to the  
261  $K_d$  value of NFO4 for OTA. We consider that with this test the LOD for OTA is at  $1.25 \mu\text{g l}^{-1}$   
262 and that differences in OTA concentration can be discriminated between  $1.25$  and  $10 \mu\text{g l}^{-1}$ .  
263 Cross-reactivity measurement of the peptide test was carried out using OTB (Fig. 4A). The

264 OTB concentration inducing 50% of the maximum possible decrease of the light signal was  
265  $8.5 \mu\text{g l}^{-1}$ , indicating that the affinity of NFO4 for OTB is circa 3-fold lower than for OTA. In  
266 addition, free OTB was a worse competitor than OTA for decreasing OTA-HRP signal.  
267 Maximal decrease reached 69% instead of 100% for concentrations above  $15 \mu\text{g l}^{-1}$ . At  $10 \mu\text{g}$   
268  $\text{l}^{-1}$ , OTA depleted the signal by 89%, whereas OTB reduced it by 47% clearly indicating that  
269 OTA detection was better than OTB with this system. Next, we evaluated whether our system  
270 could detect OTA from red wine samples. The same range of OTA concentrations was added  
271 to red wine samples. The resulting competition curve was compared to that established with  
272 pure OTA or OTB in PBS (Fig. 4A). Wine OTA could nicely be detected by the system, with  
273 a slight reduction in efficacy which can easily be explained by the enhanced number of non  
274 specific compounds co-present in wine samples. Half-inhibition occurred at  $5.8 \mu\text{g l}^{-1}$  and as  
275 for OTA in PBS the inhibition was complete. The LOD for OTA in wine was  $2 \mu\text{g l}^{-1}$  (Fig.  
276 4B), which is only slightly higher than OTA in PBS. These results suggested that the NFO4  
277 peptide can be used for detection of OTA in red wine matrices.

278

#### 279 4. Conclusions

280 The European Union (EU) has defined regulatory limits for OTA, *i.e.*,  $10 \mu\text{g l}^{-1}$  in dried vine  
281 fruits and instant coffee,  $5 \mu\text{g l}^{-1}$  in cereals and roasted coffee and  $2 \mu\text{g l}^{-1}$  in wine. Here we  
282 present a new strategy for detection of this important mycotoxin in various matrices like red  
283 wine. We have selected small peptides (12 amino acids) allowing specific recognition of  
284 OTA. The peptide named NFO4 was selected in HPLC for its higher affinity for OTA. We  
285 have validated this result by a peptide-based competitive ELISA in phosphate buffer saline  
286 and in red wine samples. The peptide-based competitive ELISA showed that NFO4 can  
287 discriminate a contamination of  $2 \mu\text{g l}^{-1}$  of OTA in red wine (without preconcentration of the  
288 sample on immunoaffinity column). This preliminary study highlights the possibility of using  
289 small peptides in biosensor systems (e.g. by electrochemical detection). Modifications of  
290 NFO4 peptide sequence may be required in order to further decrease the observed cross-  
291 reactivity with OTB which is potentially related to the phenol moiety of OTB. Such a  
292 structure-activity relationship study may increase the LOD to the lower value of  $1 \mu\text{g l}^{-1}$   
293 which is the world limit for OTA in red wine. In any case, these preliminary data are quite  
294 encouraging and strongly suggest that further work on NFO4 will allow the development of a  
295 more sensitive system, either by peptide modification or by OTA preconcentration by an  
296 affinity column.

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368

### 369 **Figure captions**

370

371 **Figure 1:** (A) Chemical structure of Ochratoxin A mycotoxin. (B) Amino acid sequence of  
372 NFO4. Single letter code.

373

374 **Figure 2** : HPLC-based peptide-based binding assays. (A) Representative HPLC profiles of  
375 three reaction media corresponding to NF04 incubation with lactic acid as negative control  
376 (left panel), hydroquinone as positive control (center panel) and OTA (right panel). Lactic  
377 acid is not detected on the HPLC profile because of lack of absorption at 230 nm. Complexes  
378 between NF04 and compounds are not detected on HPLC profiles. (B) Representative HPLC  
379 profiles of three peptides incubated with OTA: NFO1 (left), NFO2 (middle), and Chim1  
380 (right). Peak peptide depletions according to control without OTA (not shown) are 7%  
381 (NFO1), 35% (NFO2) and 10% (Chim1).

382

383 **Figure 3.** Principle of competitive immunoassay with conjugated OTA. (A) The plate is  
384 coated with the peptide NFO4. (B) The peptide is then placed in contact with the sample. If  
385 the sample contains the specific OTA, the toxin links to the specific peptide and the detection  
386 element conjugated with the toxin (usually HRP). (C) The amount of HRP-conjugated toxin  
387 that can be fixed is inversely correlated with the amounts of toxin present in the sample. (D)  
388 The non-fixed compounds are rinsed away before adding a developing product.

389

390 **Figure 4.** (A) Peptide-based competitive ELISA calibration curve. The x-axis represents the  
391 calibrator concentration of mycotoxin (OTA or OTB).  $B$  and  $B_0$  represent the bound enzyme  
392 activity measured in the presence or absence of competitor, respectively. Data are average  $\pm$   
393 standard deviation, and were fitted by decreasing exponential functions  $y=y_0 + a.e^{-bx}$ .  $Y_0$   
394 values were  $<10$  for OTA (wine and PBS) and  $>30$  for OTB. (B) Peptide-based competitive  
395 ELISA with OTA in wine. The negative control is the luminescence emitted with OTA-HRP  
396 without NFO4 peptide. Data are the mean of  $n=3 \pm$  standard deviation.


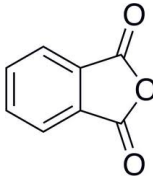
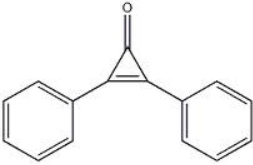
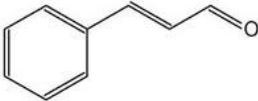
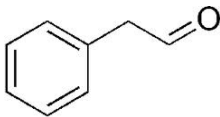
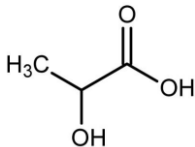
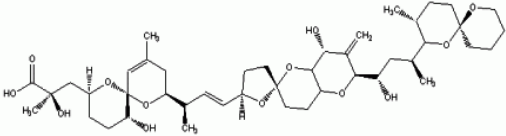
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398

**Table 1a:** HPLC-based peptide binding assays. Percentages of peak area depletion are noted. ‘100’ corresponds to 100% binding of peptide to indicated chemical compound. ‘0’ corresponds to a lack of interaction between peptide and organic compound.

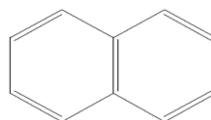
<b>Peptides/chemical compounds</b>	<b>NFO1</b>	<b>NFO2</b>	<b>NFO3</b>	<b>NFO4</b>	<b>Chim1</b>
<b>Hydroquinone</b>	100	90	100	100	80
<b>Phthalic anhydride</b>	95	100	100	100	85
<b>Diphenylcyclopropanone</b>	0	98	100	99	85
<b>Cinnamic aldehyde</b>	55	35	100	100	90
<b>Phenylacetaldehyde</b>	80	20	35	100	55
<b>Lactic acid</b>	0	0	0	0	0
<b>Okadaic acid</b>	0	0	0	0	0
<b>Naphtalene</b>	0	0	0	0	0

**Table 1b:** Chemical structures of organic compounds studied in HPLC-based peptide binding assays.

Name	MW	Chemical structure	References
Hydroquinone	110.11		Belchik et al., 2011
Phthalic anhydride	148.10		Quartier et al., 2006
Diphenylcyclopropenone	206.25		Ryan et al., 2000
Cinnamic aldehyde	132.16		Cocchiara et al., 2005
Phenylacetaldehyde	120.15		Chen et al., 2011
Lactic acid	90.08		Shen et al., 2012
Okadaic acid	805.00		Franchini et al., 2010

Naphtalene

128.17



Girschikofsky  
et al., 2012

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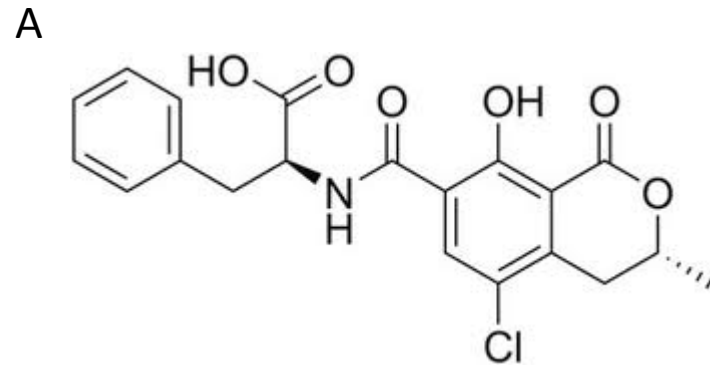


**Table 2:** Peptide binding assay. Percentages of peak area depletion are noted. ‘100’ corresponds to 100 % binding of peptide to organic compound. ‘0’ corresponds to a lack of interaction.

<b>Peptides/chemical compounds</b>	<b>NFO1</b>	<b>NFO2</b>	<b>NFO3</b>	<b>NFO4</b>	<b>Chim1</b>
<b>OTA</b>	7	35	40	70	10
<b>Hydroquinone**</b>	100	90	100	100	80
<b>Lactic acid*</b>	0	5	4	7	1

\*\* Positive control ; \* Negative control

Figure 1



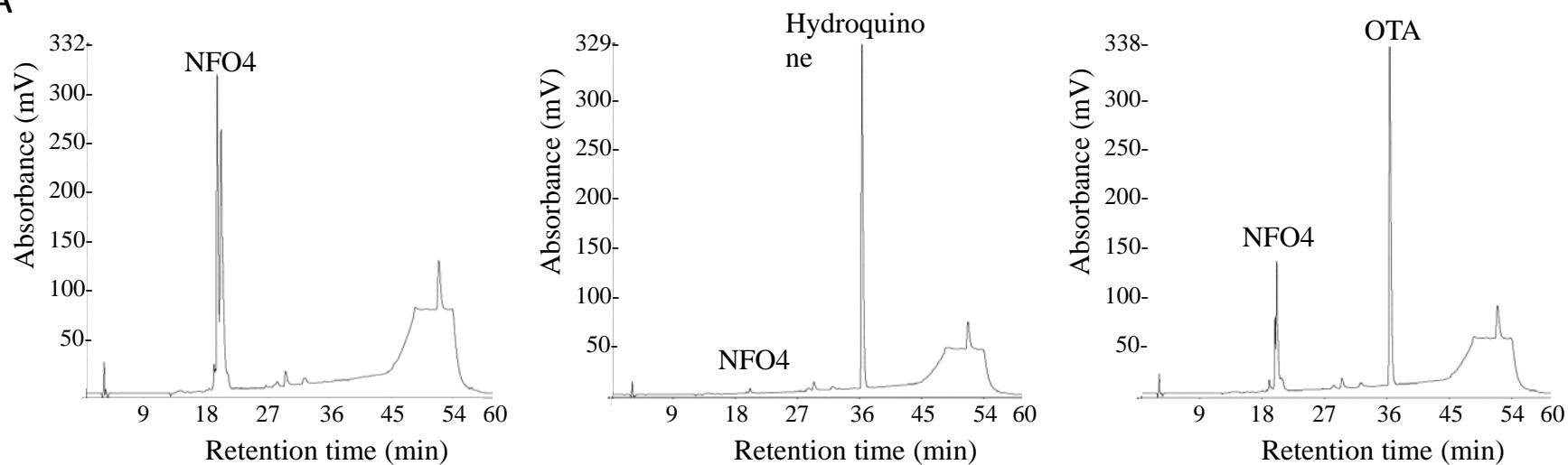
B

NFO4 primary structure

VYMN<sub>1</sub>R KY<sub>5</sub>YK<sub>10</sub>C CK-NH<sub>2</sub><sub>12</sub>

**Figure 2**

**A**



**B**

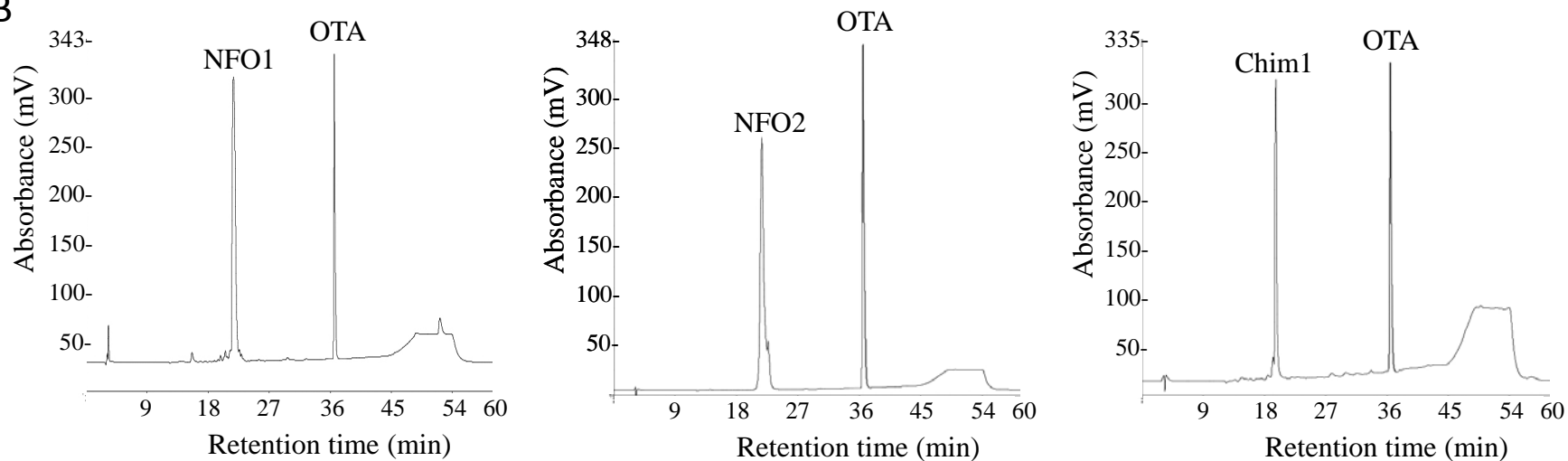
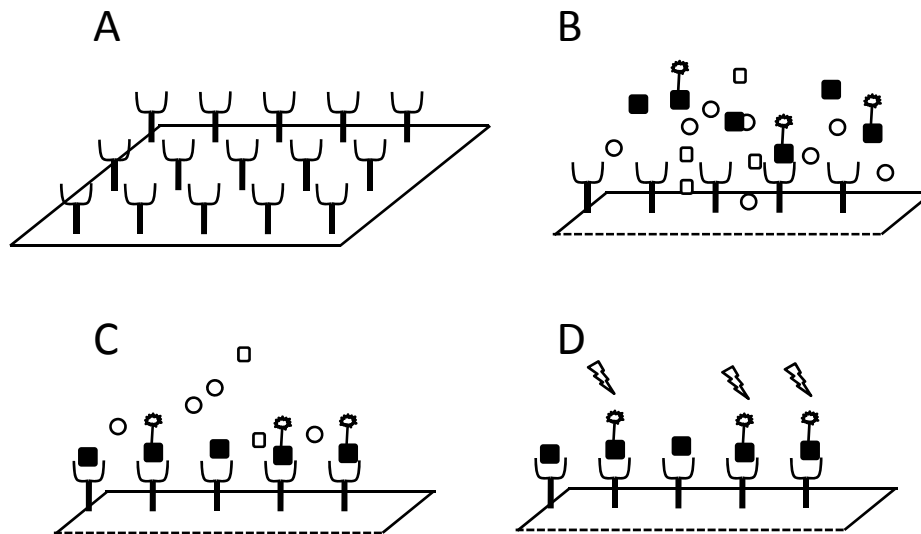


Figure 3








-  Peptide NFO4 anti-OTA
-  OTA
-  Other compounds (interferences)
-  OTA-HRP
-  Developer (substrate)

Figure 4

