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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^α -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 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 5th 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^α -(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^α -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/ H_2O /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 H_2O and freeze dried. The

131 crude peptides were purified to homogeneity by reversed-phase high pressure liquid
132 chromatography (HPLC) (C₁₈ 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/H₂O
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 C₁₈ reversed-phase HPLC (C₁₈ 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 C₁₈ reversed-phase
147 HPLC (C₁₈ 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/H₂O (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 Na₂CO₃, 35 mM NaHCO₃, 0.2 g/L NaN₃, 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 μl of OTA-HRP (horseradish peroxidase) were added
166 in each well combined with 50 μl 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 μl 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 μL
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% - 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: R^1COOR^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 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 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 B₀ represent the bound enzyme
392 activity measured in the presence or absence of competitor, respectively. Data are average ±
393 standard deviation, and were fitted by decreasing exponential functions $y=y_0 + a.e^{-bx}$. Y₀
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 ± standard deviation.


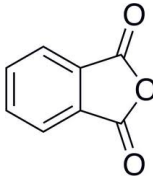
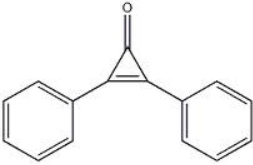
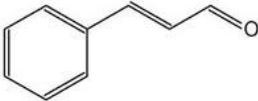
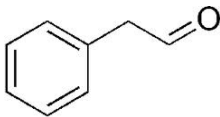
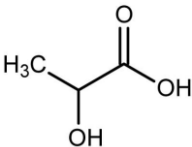
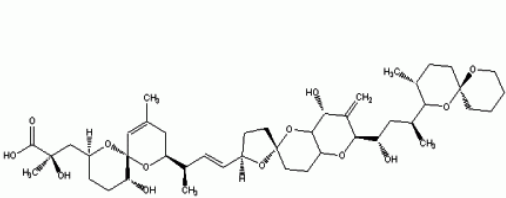
397

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.

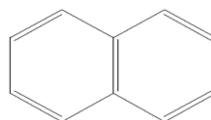
Peptides/chemical compounds	NFO1	NFO2	NFO3	NFO4	Chim1
Hydroquinone	100	90	100	100	80
Phthalic anhydride	95	100	100	100	85
Diphenylcyclopropanone	0	98	100	99	85
Cinnamic aldehyde	55	35	100	100	90
Phenylacetaldehyde	80	20	35	100	55
Lactic acid	0	0	0	0	0
Okadaic acid	0	0	0	0	0
Naphtalene	0	0	0	0	0

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



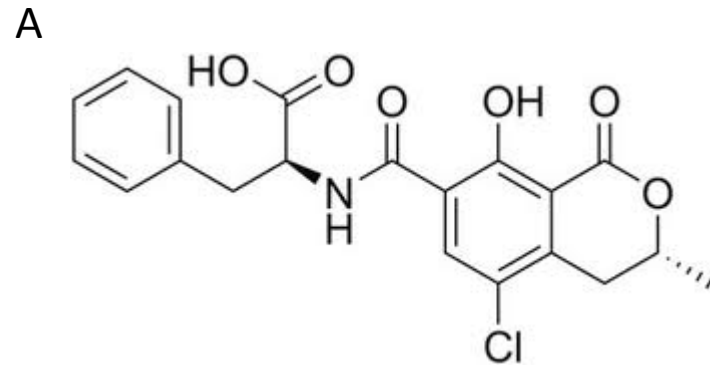
Girschikofsky
et al., 2012

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.

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

** Positive control ; * Negative control

Figure 1



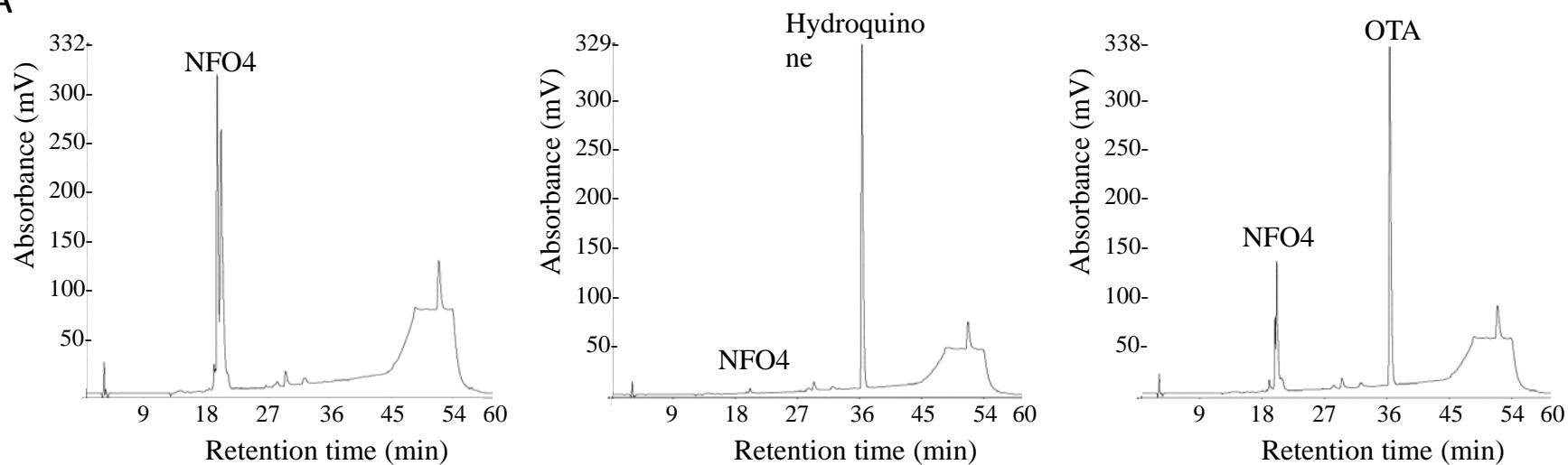
B

NFO4 primary structure

VYMN₁R KYYK₅C CK-NH₂_{10 12}

Figure 2

A



B

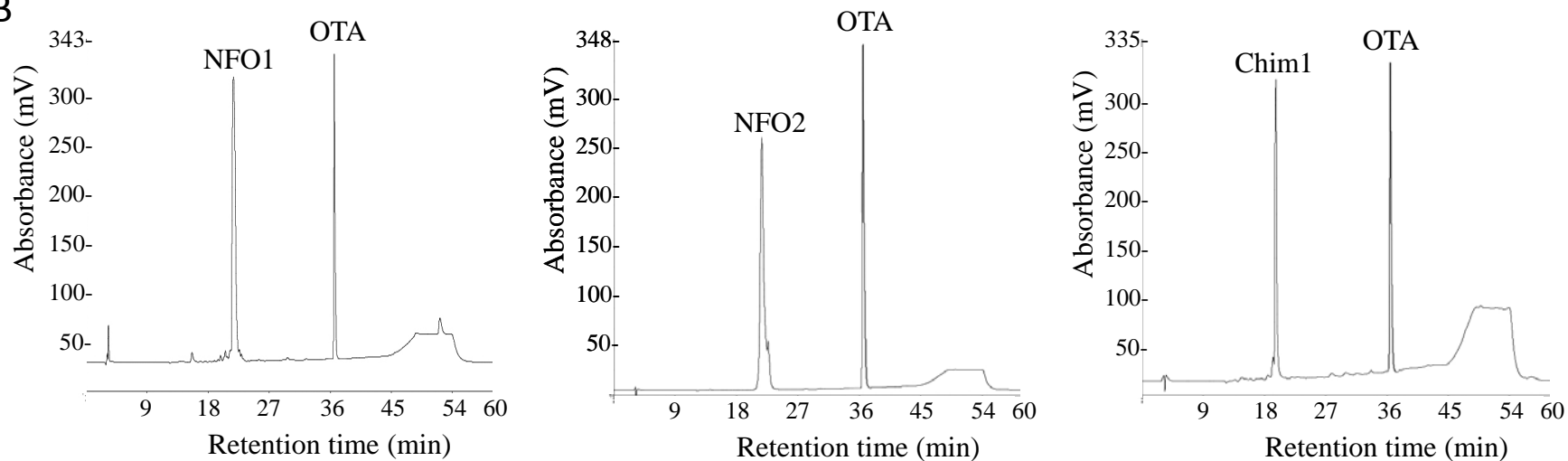
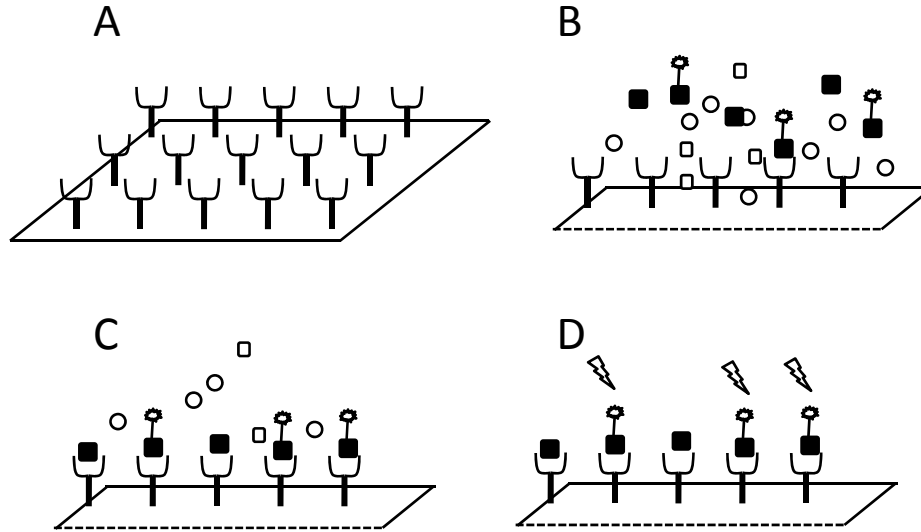


Figure 3








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

Figure 4

